Inhibition of Glycogen Phosphorylase in the Context of Type 2 Diabetes, with Focus on Recent Inhibitors Bound at the Active Site

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Abstract: Among the variety of approaches for pharmacological intervention in T2DM, the inhibition of GP with the aim of reducing hepatic glucose output is a validated and thoroughly investigated strategy. Both the academia and health companies participate in the search of potent inhibitors, that might be suitable for long-term treatment. As several inhibitory sites have been identified for GP, interest focuses mainly on structures that can bind at either the catalytic, the allosteric, or the new allosteric sites. Glucose-based motifs and azasugars that bind at the active site constitute the most populated class of GPis. During the last two years, significant progresses have been made, since newly proposed motifs have K_i values in the low micromolar and even sub- micromolar range. Without ignoring previously reported structures, new series based on β-D-glucopyranosyl-pyrimidine, D-glucopyranosylidene-spiro-isoxazoline and D-glucopyranosylidene-spiro-oxathiazole motifs appear promising. A representative from this last series, with a 2-naphthyl residue was identified as the most potent GPi to date (K_i = 0.16 μM). While no inhibition was found for sulfonium analogs, D-DAB remains the best inhibitor among five and six-membered iminosugars that showed inhibitory properties toward GP. A study of glucagon-induced glucose production in primary rat hepatocytes has suggested that amylo-1,6-glucosidase inhibitors in combination with GPis may lower glucose level in T2DM. Considering the limitations found for other potent GPis binding at other sites and the complexity of pharmacological development, the potential of glucose-based GPis is still not established firmly and more tests with cells, tissues, animals are required to better establish the risks and merits of these structures, as antidiabetic drugs. Further studies might also confirm other directions where glucose-based GPis could be useful.

Keywords: Diabetes, enzymology, glucose-based inhibitors, glycogen phosphorylase, glycomimics, hyperglycemia, inhibitors, type 2 diabetes mellitus.

Dr. Nikos G. Oikonomakos sadly passed away on the 31st of August 2008. This paper is dedicated to his memory.

I. INTRODUCTION

General Context

Nowadays, diabetes mellitus, which encompasses type 1 and type 2 as the two major forms, is an acute and global health concern. The hallmark of type 1 diabetes (T1DM) is the autoimmune-mediated destruction of insulin producing β-cells in the pancreas, resulting in absolute insulin deficiency. In contrast, type 2 diabetes (T2DM) or non-insulin dependent diabetes mellitus (NIDDM) is characterized by two defects: relative insulin deficiency and liver and peripheral insulin resistance [1]. T2DM which accounts for 90 to 95 % of the diabetic cases is a multi-factorial disease of largely unknown etiology involving both genetic [2-4] and environmental factors [5] and it is closely associated to the metabolic syndrome [6, 7]. The worldwide prevalence of obesity and diabetes has increased substantially in recent decades, with an estimation of 300 million and 170 million people, respectively, for 2006, and a prediction of 300 million diabetic patients by 2030 [6]. The rising incidence of these pathologies has grown to alarming levels in developing countries [8], including young adults as well as children, now concerned by the appearance and spreading of T2DM [9]. Diabetes has become a major health problem for most of the world's population and the coming decades must face severe health service burdens as the chronic hyperglycaemia of diabetes is associated with long-term damage, dysfunction and failure of various organs, especially the eyes, kidneys, nerves, heart and blood vessels. So as to minimize such health-threatening complications, patients with T2DM must control their glycemia by intensive lifestyle intervention as a primary treatment, with adequate diet and exercise [10], associated to pharmacological therapies. Several oral hypogly-

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cemic agents (biguanides, sulfonylureas, thiazolidinediones, α-glucosidase inhibitors) are now commonly prescribed as symptomatic treatments for normalizing physiological blood glucose levels [11, 12]. However, these drugs may induce undesirable side effects, such as hypoglycemia, weight gain, intestinal disorders [13], and being inadequate for 30-40% of patients, combination therapy for the treatment of T2DM has become quite prevalent. With increasing severity of diabetes, insulin administration is prescribed and many patients progress to insulin therapy with time [12]. In spite of substantial improvements in the management of diabetic pathologies, the limitations of symptomatic treatments are incentive for research efforts towards new therapies.

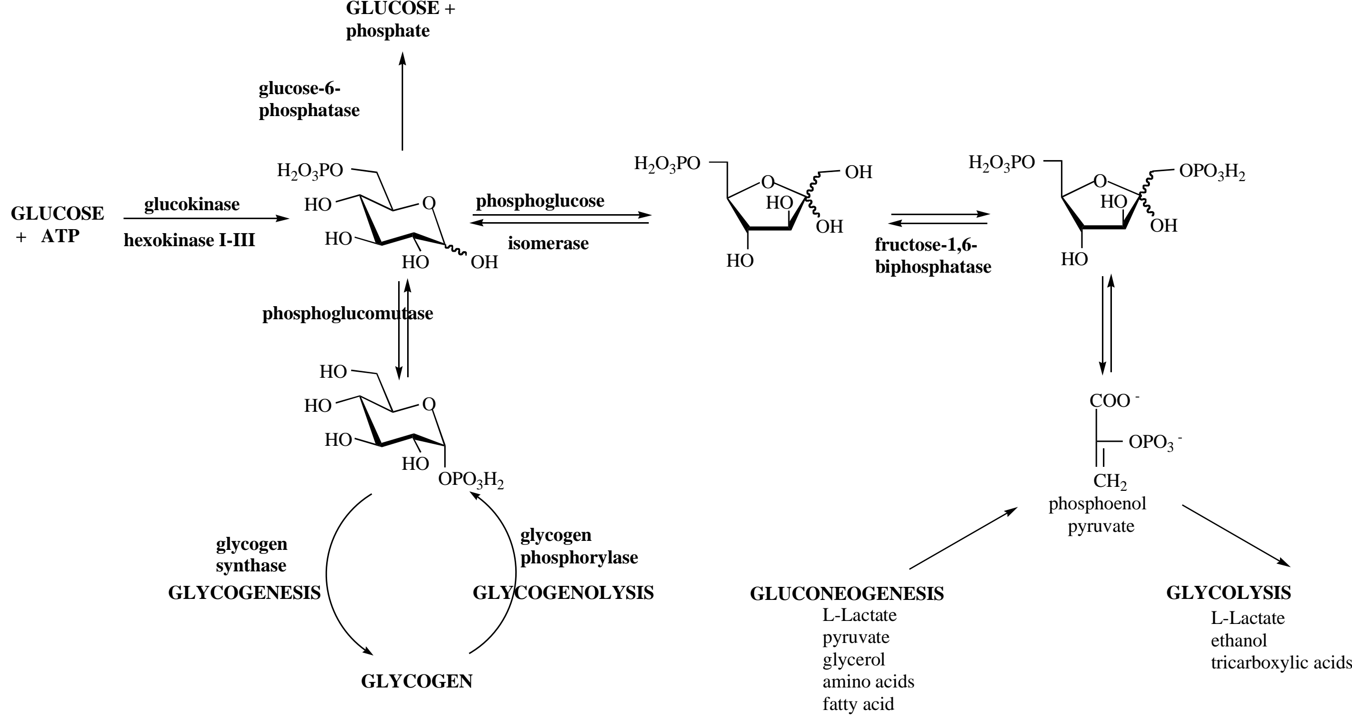
Different Pharmacological Targets for T2DM Control

For stabilizing glycemia around ~6 mM (110 mg/dL) [13] despite irregularities in food intake and energy expenditure, the regulation of glucose utilization and homeostasis is highly complex [12]. In post prandial phases, glucose resulting mostly from hydrolysis of oligo-, and polysaccharides by digestive enzymes (glycosidases), is transferred to the blood stream and distributed to such tissues as liver (storage and glucose reservoir) [14], muscles (storage and utilization) [10], and brain. The latter has comparatively predominent needs for glucose under standard conditions [15]. In line with the fact that diabetes can be induced in animals by puncture of the floor of the fourth cerebral ventricle (piqûre diabétique) as noted by the french physiologist Claude Bernard (1813-1878) [16], recent evidence suggest the key role of brain in the control of both body fat content and glucose metabolism [17], and the importance of the gut-brain axis in the control of glucose homeostasis [18]. The fat tissues regulate insulin sensivity through circulating free fatty acids derived from adipocytes which also produce a number of hormones collectively called adipokines influencing the metabolism and energy expenditure [12]. Peroxisome proliferator–activated receptor (PPAR)γ, predominantly expressed in adipose tissue as regulator of adipocyte differenciation and modulator of fat cell function are targeted by thiazolidinediones that improve glucose utilization by enhancing the response of target tissues to insulin action, without stimulating insuline release [19, 20]. Control by pancreatic hormones insulin and glucagon [21], associated to hypo- and hyperglycemic effects, respectively, is well-known. Insulin which promotes, through complex signaling cascades [22], the synthesis and storage of carbohydrates, lipids and proteins, while inhibiting their degradation and release into the circulation, is considered as the most potent hormone known [1]. Therefore, any deficiency affecting such regulations may result in disorders [13]. As the precise cause(s) of T2DM remain essentially unknown, research efforts are not focused, but encompass various targets in different organs linked to the metabolic syndrome and T2DM [12, 20, 23-25]. The novel «second-generation» approaches for the control of T2DM [26] concern glucagon-like peptide-1 [27], inhibitors of dipeptidyl peptidase IV [28] and of protein tyrosine phosphatase 1B (PTP1B) [29, 30], dual agonists of peroxisome proliferator-activated receptor α/γ [19], and liver-selective glucocorticoid antagonists [31]. The evaluation of potent, selective sodium-dependent glucose cotransporter2 (SGLT2) inhibitors, designed to reduce glucose reabsorption by the renal cortical tubule, resulting in a higher glucose urinary excretion, is also under investigation, and a number of patented *C*-aryl glycosides are under clinical trials [32, 33]. Intervention on hepatic glucose production offers also therapeutic possibilities. Data available on newly approved treatments and the targets currently being studied for the treatment of T2DM have been reviewed [34].

Hepatic Glucose Production and Possible Targets for its Control

The importance of the liver in the disposal of meal glucose and its primary role as the body's reserve supply of glucose were also recognized by Claude Bernard [35]. In the liver and muscles, available glucose may be stored as polymeric glycogen (glycogenesis, by action of glycogen synthase - GS) that can be mobilized depending on the needs. The specific features of glycogen metabolism in the liver have been reviewed [36]. Depolymerization of glycogen by phosphorolysis of an α-1,4-glycosidic bond [glycogenolysis: $\text{glucose}_n + \text{Pi} \rightarrow \text{glucose}_{n-1}$ + glucose-1-phosphate - see section **III** for other details] is catalyzed by glycogen phosphorylase (GP). In the muscle, glucose-1-phosphate is utilized *via* glycolysis to generate metabolic energy, while in the liver, by action of phosphoglucomutase and glucose-6-phosphatase, glucose-1-phosphate can be converted *via* glucose-6-phosphate to glucose. Glucose-6-phosphate is also the end-product of the hepatic gluconeogenesis pathway, which, when needed, supplies glucose from C-3 precursors. Therefore, gluconeogenesis and glycogenesis are interconnected and the three pathways, glycogenolysis included, are not independent (Scheme **1**).

Glycogenesis and gluconeogenesis being linked, estimating the contribution of the latter to hepatic glucose production is not straightforward [14, 37]. Earlier studies concluded that glycogenolysis may account for 60% to 70% of the hepatic glucose production (HGP) both in healthy controls [38] and in patients with T2DM [39] but depending on the experimental design and methodology, the estimated contribution of hepatic glycogenolysis to the total glucose production during the first day of starvation varies from 40 to 80% [36]. Data collected by various methods after overnight fasting for patients with T2DM showed that glycogenolysis contributes to 31 - 45% (with 22, and 65% as the only values out of range) [40]. There still exist unanswered questions regarding the relative contribution of glycogenolysis and gluconeogenesis to glucose production [37], in particular for people with severe T2DM [40]. A striking decrease of blood glucose during the day, even when the diabetic subjects are fasting, has been noted and analyzed in the light of various circadian rhythms [37, 40]. In healthy people, this variation appears to be minor. The hyperglycemia in people with T2DM has been attributed to an increased hepatic glucose output, and to an increased gluconeogenesis. A critical analysis indicated this is only the case in people with rather high fasting glucose values and a greatly impaired ability to secrete insulin [40]. In fact, the 20 – 40% morning increase in endogenous glucose production (EGP) vary with the patient population [37]. In the fasting state, EGP corresponds to HGP augmented with the glucose production from the kidney, considered as small and possibly variable [40, 41]. Uncertainties result from the experimental design used to



Scheme 1. Pathways for the primary metabolism of glucose.

ascertain glucose disappearance and EGP values, but also because the contribution of EGP to hyperglycemia is modulated by the metabolic clearance rate (MCR) that decreases with the severity of diabetes [37]. The substantial heterogeneity in the regulation of postprandial glucose metabolism in individuals with pre-diabetic states (Impaired Fasting Glucose/IFG; Impaired Glucose Tolerance/IGT) suggests differences in the pathogenesis of pre-diabetes and therefore, differences in the risk of subsequently developing diabetes [41] and/or differences in response to therapeutic agents that seek to either prevent or treat diabetes [42, 43]. The initial manifestation of insulin secretory abnormality is a loss of "first-phase" insulin secretion [13]. Difficulties in analyzing HGP led to varying conclusions. Initially, increased rates of gluconeogenesis and the lack of suppression of glycogenolysis were proposed as contributors to excessive glucose production in T2DM [43]. Later, hepatic insulin resistance was considered as mild while the primary cause of postprandial hyperglycemia was extrahepatic insulin resistance with accompanying defects in insulin secretion [13, 44]. Eventually, it was reported that the suppression of EGP was slightly delayed and, due to defects in insulin secretion, insulin action and glucose effectiveness [13], the glucose disappearance was substantially lower in diabetic subjects during the first 3h after meal ingestion [42]. The fact that, irrespective of glucose concentrations, MCR remains constant and low in T2DM is likely an expression of impairments in homeostasis and suggest that *low glucose clearance, which does not respond normally to metabolic stimuli, remains a fundamental lesion in the pathophysiology of T2DM* [37].

Metformin and biguanides which inhibit hepatic gluconeogenesis [45] are effective anti-hyperglycemic drugs that prove the interest of reducing HGP, and despite the uncertainties mentioned before, other targets linked to hepatic glucose production are focused by continuing investigations. Hence, glucokinase, which catalyzes the first step in glucose metabolim, the glucagon receptor, and enzymes of gluconeogenesis and/or glycogenolysis such as glucose-6-phosphatase [46], fructose-1,6-biphosphatase and glycogen phosphorylase are among the targets within the liver being investigated for development of antihyperglycemic drugs for T2DM [47-49]. Amid them, GP, the rate-limiting enzyme of glycogen degradation, is receiving a closer attention, partly because a study by Pfizer identified an inhibitor (CP-91149) as an orally active pharmacological agent that can both inhibit hepatic glycogenolysis and lower plasma glucose levels in diabetic rodents, thus demonstrating that glycogen phosphorylase inhibitors (GPis) may be useful in the tratment of T2DM [50].

II. GLYCOGEN PHOSPHORYLASE

Discovered in 1936 by Carl and Gerti Cori [51], GP (E.C.2.4.1.1) is an archetypal control protein regulated by allosteric effectors and by reversible phosphorylation [52]. First discovered in GP, control by phosphorylation is now known to be a ubiquitous intracellular process for regulations, controls and signalling [53].

GP Isoforms

GP isozymes have been identified and characterized in a large number of organisms (bacteria, fungi, yeast, plants, insects, animals) and in mammalian tissues, where GP is expressed mainly in the muscles, liver and brain, while other adult tissues express a mixture of all three types of phosphorylase in proportions that vary depending on the species. GP*a* and GP*b* represent respectively the phosphorylated (ac-

tive) and unphosphorylated (less active – vide infra for details) isoforms, and early works in the field have been compiled in comprehensive reviews [52, 54, 55]. The isozymes are named according to the animal (usually rabbit, rat, pig) and tissue from which they are extracted (eg rabbit muscles/RM; rat liver/RL). For accessibility reasons, RMGP is currently used, but investigations with the real human targets are possible since human muscle GP (HMGP) can be purified and crystallized [56] while cloning and expression of human liver (HLGP) [57] and brain [58] isoenzymes have been achieved. Crystal structure of GP isozymes from rabbit muscle (RMGP*a*) [59], human liver (HLGP*a*, HLGP*b*) [60], and human muscle HMGP*a* with bound glucose and AMP [61] are known, as well as several complexes of RMGP*b* with inhibitors bound at various sites. This classification appears oversimplified, as the brain isoform (BGP or GPBB) [62, 63] is present in embryonic tissues (fetal isozyme) [64] and in considerable amounts in human heart muscle. In this tissue, there also exists a form of GP*b*, which is a dimeric hybrid between muscle and brain isozymes [65]. Interestingly, GPBB was considered the most sensitive marker for diagnosis of acute myocardial infarction and suggested as a promising marker for the detection of ischaemic myocardial damage [62]. In another study, no BGP expression was seen in the normal human large intestine remote from cancer foci, but the BGP molecule was localized in colorectal carcinoma, adenoma, and, around carcinomas, in normal mucosa. Therefore, BGP was suggested as a novel biomarker for carcinogenesis in both the pathways of adenoma-carcinoma sequence and the de novo colorectal carcinoma [63], and for the classification of intestinal and gastric type carcinoma of the human stomach [66].

Crystallography of purified and crystallized samples support the view that all GP-s are dimers of two identical subunits isoenzymes (Mr ~ 97 500 Da), associated with an essential cofactor, pyridoxal 5'-phosphate (PLP), linked *via* a Schiff base to a lysine residue (Lys680) in each subunit [52]. For muscle GP*a*, a tetramer has been observed and crystallized [67, 68]; the dimer-tetramer interconversion of GP*a* has been investigated in the presence of caffeine [69], Glc-1-P and glycogen. Both the dimeric and tetrameric forms can bind glycogen, but the GP*a* tetramer has a lower affinity and is catalytically inactive [70]. The human GP homodimers are formed from monomers of 841 (muscle), 846 (liver), and 862 (brain) residues [60]. These isozymes (842 monomers for RMGP) have been compared, based on both the amino acid sequence and the nucleotide sequence of rat, rabbit and human muscle phosphorylase cDNA, showing homology ranging from 70, to 90 or 96% depending on the sequences considered [55, 64]. Human muscle, brain, and liver phosphorylase sequence are more conserved in the N-terminal domain than in the C-terminal domain, and the three human phosphorylase isozymes can be aligned without insertions or deletions so that amino acids 1 to 830 match. The liver protein sequence is 80% identical to either the brain or the muscle sequence, while the brain and muscle are 83% identical. Thus, the sequence of the human brain and muscle phosphorylase isozymes are more closely related than either are to the liver isozyme [55, 60].

Binding Sites of GP

Data gained from kinetic measurements and other investigations could be rationalized on a structural basis upon crystal analysis of complexes produced by co-crystallisation of GP with various ligands (eg HLGP*a*/AMP; HLGP*a*/1-GlcNAc) [60], as detailed in a number of reports or comprehensive reviews [52, 55, 71]. Despite its large size, the molecule is compact with two major excursions which form the contacts with the other subunit, close to the PLP site. PLP is the supplier of the phosphate residue needed, in close interaction with the substrate phosphate, when depolymerization of glycogen takes place at the nearby active or catalytic site [72]. More published details about this thoroughly investigated site can be found below and in other contributions in this issue. Besides Ser14 which undergo reversible phosphorylation, the other sites and their ligands are as follows: the catalytic site [72] binding substrates (glycogen and Glc-1-P) and inhibitors (glucose and glucose-analogs), the allosteric site that binds the activator AMP and the inhibitor Glc-6-P [68], the inhibitor site (also termed nucleoside site or purine binding site) that binds purines (eg adenine and caffeine), nucleosides (eg adenosine and inosine), nucleotides (eg AMP, IMP, and ATP), NADH, FMN, FAD, and riboflavin [71, 73, 74], and a novel allosteric inhibitor site or indole carboxamide site, located at the subunit interface, in the region of the central cavity [75, 76]. Located close to the active site, approximately 10 Å away, the purine inhibitor site exists as a slot formed by parallel "stacking" of the hydrophobic side chains of Phe285 and Tyr613. No physiological role has yet been established for the nucleoside inhibitor site and the indole inhibitor site. More recently, a novel binding site, located at the protein surface, far removed (~ 32 Å) from the other binding sites was discovered [77]. The glycogen storage site that insures a close association of polymeric glucose with the GP enzyme is on the surface of the molecule approximately 30 Å from the catalytic site, 40 Å from the original allosteric site, and 50 Å from the new allosteric site [52]. The storage site has been shown crystallographically to bind different glucose oligomers, as maltopenta-, and -heptaoses, and β-, and γ-cyclodextrines, these cyclic analogs having K_i values (respectively 14.1 and 7.4 mM) lower than that of α-cyclodextrine (K_i = 47.1 mM), which was not observed bound in the crystal of RMGP*b* [78]. The affinity of glycogen for this site is estimated 20-fold higher than that for the active site [72]. *In vivo* a substantial proportion of GP is associated with glycogen particles, approximately 400 Å in diameter, that also contain other enzymes involved in glycogen regulation *via* coordinated control. The storage site also plays a role in regulatory properties though reciprocal control with GS (vide infra) and activation mechanisms. One of them originate from the aggregation properties of GP, in which activation by either phosphorylation or AMP leads to a change in association of the subunits from dimers to less active tetramers. Glycogen promotes dissociation of tetramers to dimers through competition for binding at the storage site [52, 70]. While all GP-s consist of a conserved catalytic core, close comparison of yeast and muscle GP*b* has revealed changes over the course of evolution from yeast to vertebrates: the Glc-6-P binding site was modified to become a bi-functional switch [68], while phosphorylation concerns different sites [Thr(-10) in yeast, Ser14 in mammals] both

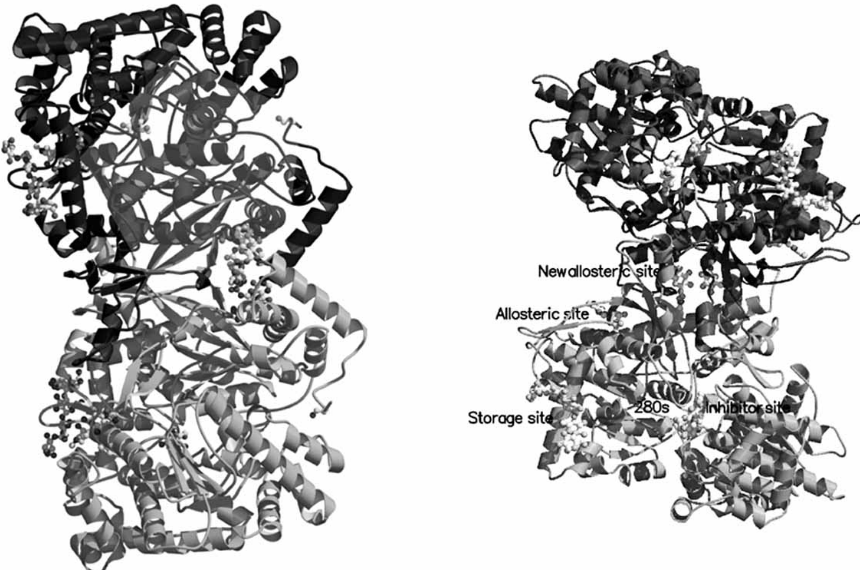


Fig. (1). Crystal structure of GP*b* homodimer showing the main sites, in particular the tower helix and the Ser14 phosphorylation site, near the NH_2 terminus of the peptide chain (left view); after rotation by 90° along a vertical axis (right view), the tower helix and the 280s loops are visible at the front, as well as the allosteric, new allosteric, inhibitor and storage sites. The catalytic sites, close to the inhibitor sites, are at the center of each subunit.

(Figures kindly sent by E. D. Chrysina (IOPC, NHRF, Athens).

located near the subunit interface [79]. As outlined below, the signals from the phosphorylation and AMP sites induce changes in terms of structure (conformation) and enzyme activity, through a relay process to the catalytic core/site [79] at the centre of each subunit and well away from the subunit-subunit interface [53].

Phosphorolysis Mechanism at the Catalytic Site

In vitro, GPs catalyse the reactions: glucose$_n$ + Pi → glucose$_{n-1}$ + glucose-1-P, in either direction depending on whether inorganic phosphate (Pi) or Glc-1-P is provided as substrate [79]. Therefore, depending on conditions, GP can promote phosphorolysis of α-1,4-glycosidic bond at the non-reducing end of glycogen, or glycogen synthesis. These reactions which occur with retention of the α-configuration are thought to proceed *via* carbenium ion intermediate, since no evidence indicate formation of covalently bound transients [72]. The catalytic site is a deep cavity 15 Å from the protein surface, accessible *via* channel 1 (or channel α) after movements of the 280s loop and Arg569 [53, 71]. The phosphorolysis mechanism has been probed by crystallographic binding studies, and since glycogen is too large to diffuse into phosphorylase crystals, a small molecular weight substrate heptenitol **3** (Table **1**) was used instead [80]. Heptenitol is a 7-carbon glycosylic substrate that is phosphorylated by GP to heptulose-2-phosphate by a mechanism that appears identical to the natural reaction from the kinetic and solution studies. It was concluded that the 5'-phosphate of PLP acts as a general acid to promote attack by the inorganic phosphate on the glycosidic bond of the terminal glucose unit in a glycogen chain (vide infra for more information on glycogen degradation). This requires direct PLP 5'-phosphate interaction with the inorganic phosphate [52, 53, 72]. The *Escherichia coli* maltodextrin phosphorylase (MalP), a non-regulatory phosphorylase that shares similar kinetic and catalytic properties with the mammalian GP was studied and crystal structures of three MalP-oligosaccharide complexes showed the phosphate group poised to attack the glycosidic bond and promote phosphorolysis [81]. Crystallographic studies with GP*b* and the potent β-glucosidase inhibitor D-gluconohydroximo-1,5-lactone *N*-phenylurethane (PUG **4**, Table **1**) have revealed channel 2 (or channel β), an extensive pocket lined by both polar and non-polar groups and oriented in the β-direction relative to the C1 of D-glucopyranosyl ring bound at the active site [82]. Studies with **4** and its precursor D-gluconohydroximo-1,5-lactone led to the conclusion that the catalytic site of GP has no great preference for a half-chair or chair conformation, and that, in contrast with the situation for β-glycoside–recognition enzymes, distortion of the glucopyranose ring toward half-chair geometry and promotion of trigonal geometry at C-1 is not an obligatory first step in the phosphorolysis reaction [83]. More insight on the phosphorolysis mechanism was obtained based on calculations and comparative kinetic and crystallographic studies of glucose-fused triazole and tetrazole [84, 85]. As explained next, GP is active provided there is accessibility to the active site, that is dependent on the conformation state of GP, in relation with the phosphorylation state and the influence of allosteric effectors.

GP Conformation and Activity States

The activation of GP by phosphorylation is the end result of one of the best understood signalling pathways [53]. Phosphorylation of Ser14 located near the subunit-subunit contact induces changes in the tertiary structure and results in conformational changes from the tense (T) state to the re-

relaxed (R) state, whereby the mobile homodimer changes its quaternary structure upon rotation of each subunit by 5°. These events are understood on a molecular basis and their sequence explains the signal transduction from the phosphorylation and allosteric sites to the catalytic site [52, 53], as pictured for the phosphorylation-initiated activation relay in yeast GP [79]. Of particular importance are residues 262 to 278 (tower helix) of each unit, which, in the T state, are antiparallel and packed with an angle of tilt of about –20°. Each helix is followed by a loop of chain termed the 280s loop (residues 282 to 286 that contains Asp283 whose side chain is directed toward the catalytic site and is linked *via* 2 water molecules to the 5'-phosphate of PLP) that blocks access for the large substrate glycogen to the catalytic site. Either phosphorylation or binding of activator AMP promote interconversion to the R state, in which the tower helices change their angle of tilt to about –70° and pull apart. As a result, the 280s loop is displaced, the catalytic site becomes accessible, and phosphorolysis possible, with the participation of basic Arg569 which helps to create the higher affinity dianion recognition site of phosphate [52, 53, 86].

While substrate glucose-1-P promotes the R state conformation, glucose-based inhibitors favour the T state, with the notable exception of D-DAB **13** (Table **1**). Caffeine, nucleosides, and other inhibitors binding at the inhibitor site promote the T state. Crystallographic studies have shown how the allosteric effector AMP site can exhibit a tight binding mode for the activator AMP in the R state structure and a tight binding mode for Glc-6-P in the modified T state (T ') structure [87]. An intermediate conformation with T state and R state features has also been observed for HMGP*a* bound with glucose and AMP [61]. In line with its inhibitory properties, glucose bound at the catalytic site favors the T state through its interactions with residues Asp283, and Asn284 which tie the 280s loop in the T state conformation. However, the tertiary structure at the Ser14 site and the subunit interactions are almost identical to those of R state GP*a* [52]. In the liver, GP*a* functions as the glucose receptor: the binding of glucose competitively inhibits the enzyme and also induces conformational changes in active GP*a* that make phosphorylated Ser14 more accessible to protein phosphatase [36], thus resulting in inactivation of the enzyme and arrest of glycogenolysis. As another important consequence, these events lead to GS activation (vide infra). The opposite situation in which GP*b* is converted to GP*a* in hepatocytes in the presence of inhibitors stabilizing the R state conformation is documented by the crystallographic analysis of the enzyme in complex with D-DAB **13** [88] and metabolic studies with cells [89, 90]. These data provided unequivocal support for the physiological role of the regulation of glycogen synthase by the concentration of GP*a*.

The physiological role of liver GP is to insure a supply of glucose for extrahepatic tissues such as central nervous system and to suppress hepatic output of glucose under conditions of high blood glucose. In muscle, the product Glc-1-P is metabolized by glycolysis to meet the energy requirements for muscle contraction. The regulatory roles of GP are controlled by phosphorylation or allosteric effectors with sub-

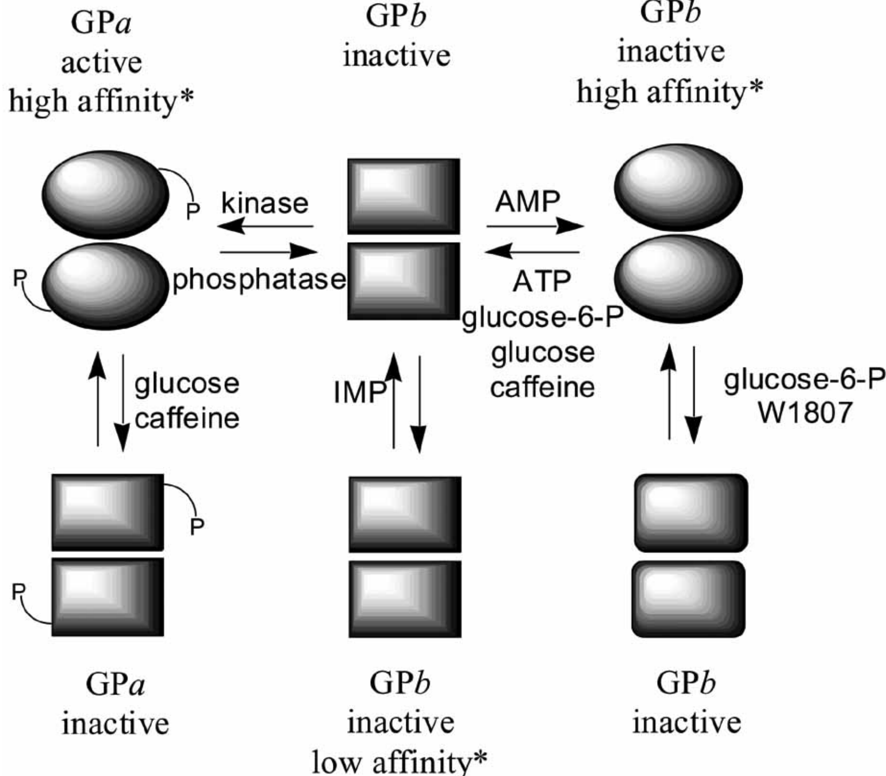


Fig. (2). Schematic representation of the allosteric transition of GP (adapted from [71]).
Tense and relaxed states are shown as squares (T), round squares (T '), and circles (R); * for substrate.

stantial differences according to isozymes origin, even though they share a 80% amino acid sequence identity [57]. Whereas AMP activates muscle GP*b* to 80% of the activity seen with muscle GP*a* (AMP also increase the activity of muscle GP*a* by a further 10%), AMP activates liver GP*b* to only 20% of the level of liver GP*a*, with no effect on liver GP*a*. Conversion of unphosphorylated liver GP*b* to phosphorylated GP*a* fully activate the liver isozyme whose maximal activity is only 34% of that of the muscle enzyme [52, 55, 91]. Thus, AMP is a potent allosteric activator of the muscle GP*b* (thus promoting the depolymerization of glycogen for glycolysis according to the energetic needs of muscle fibers under less stressful conditions – see [57] for details), whereas AMP only weakly activates the liver GP*b*, and therefore, the liver isozyme predominantly is activated through phosphorylation induced *in vivo* by hormones such as glucagon and adrenaline [53]. With insulin and glucose having deactivating effects, the primary function of liver phosphorylase is to modulate whole body glucose homeostasis. The lower specific activity of liver GP protects against inappropriately high rates of glycogenolysis that might lead to hyperglycemia; in contrast, the high specific activity of muscle phosphorylase *a* allow*s* rapid mobilization of fuel for energy production during “fight or flight” situations [57].

Control of GS by GP*a*

Glucose, as the physiological regulator of hepatic glycogen metabolism acting synergistically with insulin, promotes inactivation of GP*a*, with the result of diminished glycogen degradation and enhanced glycogen synthesis. This process involves glycogen synthase (GS), an enzyme controlled by reversible multiple phosphorylation (conversion to the inactive b form), whereas the dephosphorylated GS*a* is active. Among the specific features of glycogen metabolism in the liver [36], it is important to note that Glc-6-P is: a) an allosteric activator of GS*b*; b) a promoter of the dephosphorylation of GS*b* by glycogen-associated protein phosphatase-1 (PP1), c) having a more complex role in the regulation of glycogen metabolism [92]. Therefore, Glc-6-P mediates the glucose-induced activation of GS (whereas it inhibits GP). By converting inactive GS*b* to active GS*a*, PP1 is the key regulator of the regulation state of GS in liver cells. PP1 is a protein serine/threonine phosphatase that regulates many cellular functions through the interaction of its catalytic subunit (PP1c) with over 50 regulatory subunits [93]. A variety of PP1c-targeting subunits can target the enzyme to glycogen, as those found in the liver, R5, R6, and G_L, the most abundant. In the liver, targeting of PP1 to glycogen particle by G_L increases PP1 activity towards GS. *Via* 16 amino acids at its C-terminus, and essentially the two last tyrosines [91, 94], G_L can also interact with HLGP*a*, but not with HLGP*b* for structural reasons [95]. The result of G_L-HLGP*a* interaction is a potent allosteric inhibition of PP1, an alternative mechanism for inhibiting glycogen synthesis when glycogenolysis is activated. As PP1 in turn suppresses GP and phosphorylase kinase activities through dephosphorylation (for further possibilities in hepatocytes, see [96]) while activation of GS *via* its phosphatase (PP1) can be allosterically inhibited by binding of GP*a* to G_L [95, 97], a strong reciprocal control between GP*a* and glycogen synthase activity that is crucial for glycogen metabolim can be recognized [98].

At this point, the general features which are of importance when considering the inhibition of GP for glycaemic control in T2DM have been recalled, with the hope they could be useful for those not familiar with the field. However, a large amount of relatively recent informations is available, as research efforts from both the academia and pharmaceutical companies have revealed inhibitors with increasing affinity for the active, allosteric, and new allosteric sites of GP. The corresponding data have been compiled previously in comprehensive reviews, so readers are kindly refered to these documents [71, 99-104]. Some are mainly devoted to synthetic work, kinetic data, structural analysis, covering either all kinds of GPis [71, 103, 104] or specifically glucose-based ones [100-102]. Others, in addition to addressing the chemical and biochemical aspects, emphasize the pharmacological and physiological consequences of inhibiting glycogenolysis [99, 105], that were also discussed when other new hepatic targets for glycaemic control in diabetes were considered [34, 47, 48]. The patent literature has been also reviewed [106] showing that in recent years, inhibitors binding at the allosteric (AMP) site or the new allosteric (indole) sites have received more attention from pharmaceutical companies.

The next section will present glucose-based GPis and iminosugars binding at the catalytic site reported after ref. [104] appeared.

III. INHIBITORS BINDING AT THE ACTIVE SITE OF GP (2008 TO JUNE 2010)

The recognition that α-, and β-D-glucose **1**α and **1**β are inhibitors of GP was the starting point of the design, synthesis, and evaluation of glucose analogs and related molecules as potential inhibitors of GP that might be of interest for new pharmacological approaches of T2DM. *N*-Acetyl β-D-glucopyranosylamine **2** (Table **1**) was early identified as a potent inhibitor [107], but further studies with rat hepatocytes concluded that in the cells **2** was converted by phosphorylation into 6-*O*-phosphate of *N*-acetyl β-D-glucopyranosylamine [108] found to be a specific inhibitor of glycogen-bound PP1 [109]. Therefore, although **2** was a potent inhibitor of GP, the 6-*O*-phosphate formed was responsible for the failure of **2** to elicit GS activation in hepatocytes. Comparison of the inhibitory properties of 4 *N*- and *C*-linked C1-substituted derivatives (*N*-acetyl-β-D-glucopyr-anosylamine, *N*-chloroacetyl-β-D-glucopyranosylamine, 2,6-anhydro-D-*glycero*-D-*ido*-heptonamide, 2,6-anhydro-D-*glycero*-D-*gulo*-heptonamide) toward GP activity from crude extracts of rat liver and muscles showed **2** was the most effective, with K_i values of 51 μM (muscle GP*a*), 30 μM (muscle GP*b*), 2.7 mM (liver GP*a*), and 4 mM (liver GP*b*) [110]. The 4 analogs tested inhibited muscle GP more potently than liver GP. Moreover, compound **2** was found a moderate inhibitor of rat liver hexokinase and glucokinase, and it was concluded that more specific and more potent GPis were necessary for potential therapeutic use. Another study with two C-6 epimeric 7-deoxy-heptoses mimics of D-glucose showed their opposite effect on GS activation and levels of Glc-6-P in hepatocytes, thus shedding some insight on the complexity of phenomena in living systems, with glucose-based inhibitors [111]. As first shown by a group in Oxford [112], spirohydantoins **10** were found potent inhibitors of the different

GP isoforms [101] and, in gel-filtered rat liver extracts, spiro-thiohydantoin **10b** increased significantly the inactivation of GP and the sequential activation of GS. When administered *in vivo* into the *v. portae* of Wistar rats, **10b** was also effective and significantly decreased the activity of RLGP*a* within 5 min [113, 114].

Therefore, these encouraging results which came from early efforts in the field, called for more detailed investiga-

Table 1. Selected GPis Binding at the Active Site and Underlying Concepts for Inhibition

D-Glucose β-D-Glc **1β** K_i = 7 mM	**5** [116] K_i = 305 µM / RMGP*b*	**Spirobicyclic inhibitors**[a] **10** **a** X = O K_i = 3.1 µM / RMGP*b* [112] **b** X = S K_i = 5.1 µM / RMGP*b* [119]
α-D-Glc **1α** K_i = 1.7 mM	**Binding to β-channel** **6** [116] K_i = 5.6 µM / RMGP*b*	**11** [112] K_i = 320 µM / RMGP*b*
***N*-Acetyl-β-D-gluco pyranosyl amine**[a] His377 **2** [107] K_i = 32 µM	**Glyco-heterocycles** **7** X = NH K_i = 11 µM / RMGP*b* **8** X = S K_i = 229 µM / RMGP*b* [117]	**12** [120] K_i = 59.7 µM / RMGP*b*
Transition-state analogues **3** [52, 80]	**9** [118] K_i = 180 µM / RMGP*b*	**Imino-sugars** DAB: **13** [122] K_i = 400 nM / RMGP*a*
4 [82] K_i = 400 µM / RMGP*b*		

[a]Crystal studies of complexes with GPis bound at the catalytic site of the enzyme revealed favourable H-bondings between His377 and the NH group of **2** and **10**, a situation not observed for **5** and **6**.

tions, and during the last decade, the class of glucose analogs has been developed further, and it now gathers the larger number of representatives. As indicated by selected inhibitors (Table **1**), they can be classified according to several main types (*N*-acyl β-D-glucopyranosylamines, β-D-glucopyranosylureas, biurets, spiro-compounds, glucose-derived heterocycles, iminosugars), whose syntheses, kinetic data and crystallographic studies of complexes with ligands bound at the catalytic site of GP have been discussed previously [71, 99-105]. It was shown that all hydroxyl groups around the D-glucosyl ring are important for binding at the active site, as D-galactose or D-xylose based analogs were found less active (see [115] for recent relevant data). These studies provided a firm structural basis to explain the inhibitions observed and for the design of new ligands, according to different concepts. For example, compounds **3** [52] and **4** [82] were investigated, as their planar geometry at the anomeric center resemble transition-state intermediates of the phosphorolysis reaction. Interestingly, **3** was phosphorylated by the enzyme, and the complex with 2-phosphoryl heptulose bound at the catalytic site was subjected to crystal analysis [80]. Indeed, **3** and **4** brought new insight on the mechanism of the reaction and recognition site (vide supra) while **4** and its precursor D-gluconohydroximo-1,5-lactone were both found considerably less potent than expected for transition-state analogue [82, 83]. H-Bondings between the 6-OH and NH groups in **2** with His377 as observed also for the spiro-hydantoins **10** was an incentive to investigate related derivatives (acylureas **5**, **6** [116] and analogous derivatives). These proved to be effective inhibitors, although no H-bond between the NH (GPis) and CO (His377) was present in **5** and **6**, thus showing that the interactions, contacts and binding network to the enzyme get adapted specifically to the structure of the ligand considered. In particular, comparing the K_i for **5** and **6** shows clearly the beneficial contribution of an aryl residue when linked to glucose by an appropriate linker. This was rationalized by crystal data which proved binding to the β-channel. The 2-naphthyl group fits well this site, and in several series of glucose analogs, the one displaying a 2-naphthyl group is the best inhibitor. Compounds **7**, **8** [117] and **9** [118] are representatives of glucose-derived heterocycles in which apparently minor modifications resulted in significant changes in the binding affinity (see [77] for crystal studies with **7** and **8**). This was also apparent when comparing the K_i values for the spiro-hydantoins **10a** [112] and **10b** [119] with that of the epimer **11** [112] or the isomer **12** [120]. The data accumulated with the imino-sugars after 1,4-dideoxy-1,4-imino-D-arabinitol (D-DAB **13**) [121] was found to be the more potent inhibitor of GP (K_i = around 400 nM) led to a similar conclusion (vide infra).

The potency of **13**, irrespective of GP phosphorylation state or isoform, has attracted much interest and the inhibition of glycogenolysis in primary rat hepatocytes by **13** has been reported [121], as well as its anti-hyperglycaemic effect in ob/ob mice [122]. The effect of glucose on the potency of **13** revealed no physiologically relevant glucose dependence *in vitro* [89]. The *in vivo* effects of **13** have been reviewed [47, 105]. DAB **13**, isofagomine and its *N*-3-phenylpropyl derivative in complexation with RMGP*b* have been shown by X-ray crystallography to bind tightly in the presence of substrate phosphate at the catalytic site [88]. DAB **13** binds only in the presence of high concentrations of phosphate with the three OH groups in **13** mimicking the OH groups in the 3, 4, and 6 positions of D-glucopyranose, and the oxacarbenium ion transition state of the phosphorolysis. DAB was also shown to stabilize the R state conformation, thereby favouring phosphorylation and activation to GP*a*, and allosteric inhibition of PP1 by GP*a*, leading to decreased glycogen synthesis [90]. DAB has been used as a pharmacological tool in the investigation of the functional role of glycogen with homogenates and intact brain tissues and cells [123].

Investigations conducted in past years have revealed potent glucose-based inhibitors with K_i in the low μM range (**2**, **6**, **7**, **10a**, **10b**, **12**), or even the nanoM range for D-DAB **13** (Table **1**). Complete information can be found in recent reviews [103, 104]. More recent data published after 2008 about inhibitors binding at the active site [104] are presented below.

a. GPis of the *C*-β-D-Glucopyranosyl Type

Following previous reports on the synthesis of GPis, an Indian group has exploited the condensation of D-glucose with pentan-2,4-dione to afford β-D-glucopyranosylpropanone **14** as an access by the aldol condensation to various aromatic aldehydes to a series of butenonyl *C*-glucosides (**15a-i**), and analogs formed from D-xylose and cellobiose [124]. Michael addition of diethylmalonate led to products **16**, as *R,S* mixtures (Scheme **2**). The deacetylated compounds were evaluated at a 100 μM concentration against α-glucosidase (purified from the rat intestine), glucose-6-phosphatase, and RLGP. The inhibitions measured showed clearly that compound **15b** which bears a 2-naphthyl group is the best GP inhibitor (IC_{50} = 98.0 μM) in the series. As **15d** inhibited glucose-6-phosphatase (IC_{50} = 87.0 μM), while **15h** exhibited an α-glucosidase activity (IC_{50} = 51.5 μM) superior to that of acarbose, compounds **15b**, **15d**, and **15h** were tested for their *in vivo* effects. In sucrose loaded hyperglycaemic rats, the effects of **15b** and **15h** were slightly below that of metformin (respective activities: 25.0, 26.7, 31.2 %), while with sucrose-challenged STZ-induced diabetic rats, **15b** and **15h** showed a decline of 14.2 and 14.1 % of the hyperglycaemia, compared with 26.9% for metformin. In both tests, compound **15d** with a phenyl substituent was found less effective, compared to the other *C*-glucosyl butenonyl analogs with 2-naphthyl or 3,4-dimethoxyphenyl groups.

As cyano derivatives can be converted to the corresponding cyclopropylamines by means of $Ti(OiPr)_4$ and EtMgBr, these titanium-mediated conditions were applied to the benzoylated cyanide **19** to afford, upon migration of the 2-benzoyl group, compound **20** (Scheme **3**). Zemplén debenzoylation led to **21** which was found to be a weak inhibitor of RMGP*b* (16% inhibition at 2.5 mM), possibly because the cyclopropyl group prevents hydrogen bonding between the NH and the His377 carbonyl, as observed for the inhibitor **2** [125].

C-Glucosyl hydroquinones and analogs have been obtained upon coupling between 1,4-dimethoxybenzene and glucose penta-acetate **22**, to afford stereoselectively

D-glucose D-xylose cellobiose

a b c

14

R = Ac, H (**15 a- i**)
analogues: **17 a,h** (D-xylo),
18b,d (cellobiose)

R = Ac, H (**16a,b,d,e,h**)

	Ar	GP Inhibition[a]
β-D-*gluco*		
15a	4-chlorophenyl	-36.2
15b	2-naphthyl	-52.4
15c	4-methoxyphenyl	-37.2
15d	phenyl	+7.3
15e	3-pyridyl	-28.9
15f	4-hydroxyphenyl	-3.4
15g	3-nitrophenyl	+15.6
β-D-*gluco*		
15h	3,4-dimethoxyphenyl	-5.9
15i	3,4,5-trimethoxyphenyl	-19.6
16a	4-chlorophenyl	-5.4
16b	2-naphthyl	-14.7
16d	phenyl	-25.9
16e	3-pyridyl	-11.7
16h	3,4-dimethoxyphenyl	-24.0
β-D-*xylo*		
17a	4-chlorophenyl	-19.6
17h	3,4-dimethoxyphenyl	-21.5
β-D-*cellobio*		
18b	2-naphthyl	-8.3
18d	phenyl	-30.8

Conditions: condensation with: (a) pentan-2,4-dione, (b) ArCHO, (c) $CH_2(CO_2Et)_2$.
[a]-% activity at 100 μM concerntration

Scheme 2. GPis of the *C*-β-D-glucosyl type.

EtMgBr
$Ti(O\textit{i}Pr)_4$

19 via

path 2

R = Bz (**20**), H (**21**)

path 1

Scheme 3. 1-(β-D-Glucopyranosyl)cyclopropylamine as GPi.

compound **23**, which was subjected further to oxidation, reduction, and deacetylation (Scheme **4**). *C*-Glucosylhydroquinone **26** and –benzoquinone **27** were found to be competitive inhibitors (K_i = 0.9 and 1.3 mM respectively) of RMGP*b*, with respect to the substrate Glc-1-P. Crystal structures of **26** and **27** in complex with RMGP*b* revealed that the inhibitors can be accommodated at the catalytic site at approximately the same position as α-D-glucose, the benzo(hydro)quinone groups occupying the β-pocket and stabilizing the closed conformation of the 280s loop [126]. Moreover, other tests showed that **27** (IC_{50} = 25.6 μM) and its tetra-*O*-acetyl derivative (IC_{50} = 4.8 μM) are inhibitors of PTP1B [127]. A few sugar derivatives inhibit PTP1B, and (benzo)quinones constitute a specific group of inhibitors [128]. Interestingly, **27** resembles Carmine, a naturally-occuring *C*-glucosyl compound, used as food dye and found to inhibit moderately PTP1B (IC_{50} = 26 μM) [129], a phosphatase that intervenes at the early stage of insulin signaling [22].

b. GPis of the *S*-β-D-Glucopyranoside Type and Related Glucosides

After computations based on the 4D-QSAR methods have predicted a K_i = 0.59 μM for GP inhibition by an anomeri-

22 → (a) 23 → (b) 24 → (c) 25 → (d) 26 → (e) 27

26: K_i = 0.9 mM / RMGP*b*   27: K_i = 1.3 mM / RMGP*b*

Conditions: a - 1,4-dimethoxybenzene, Sn^{IV}; b - CAN; c - $NaBH_4$; d - MeONa in MeOH; e - Ag_2O or $PhI(OAc)_2$.

Scheme 4. *C*-β-D-Glucopyranosyl hydro(benzo)quinones as GPis.

cally bifunctionalized α-D-glucopyranosylsulfonamide with NHAc as the β-substituent, its synthesis was considered, *via* intermediates **32** and **35**, that could be stereoselectively brominated at the anomeric center, then further converted to azido and NAc derivatives. As outlined in Scheme **5**, the desired glucosyl sulfonamide **35** was prepared by *S*-glycosidation of peracetate **22** to afford **30** which, upon oxidation and DBU-induced 1,2-elimination, produced the sulfinate intermediate **34**. It was directly reacted with H_2NOSO_3H to afford **35** in good yield. Radical-mediated bromination of sulfone **32**, and sulfonamide **35** afforded low yields of the corresponding α-bromides which showed either no reaction, or decomposition upon attempted substitution by azide ion. The deacetylated products **31**, **33**, and **36** isolated in variable yields showed no inhibition of RMGP*b* [130], although 1-thio-β-D-glucopyranose **28** and its derivative **29** (K_i = 1 and 0.65 mM, respectively) were moderate inhibitors of RMGP*b* [131].

Syntheses of *O*-, *S*-, and *N*-glycosides of hept-2-ulopyranosonamides from the bromo-glycosylformamide **37** met with more success, giving access under simple conditions and in good to excellent yields, to the corresponding anomerically bifunctionalized glucose derivatives **38**, **40**, and **42** (Scheme **6**). However, after debenzoylation, no significant inhibition of RMGP*b* was found (**39**: 21% inhibition at 625 μM; **41a-c**: no inhibition at 625 μM; **43**: IC_{50} > 60 mM) [132].

c. *N*-β-D-Glucopyranosylamides, Related Ureas and Biurets as GPis

The inhibition observed for **2** has spured research efforts toward derivatives of glucosylamides, related ureas, and biurets which have been reviewed in detail [103, 104]. Structures of the glucopyranosylamide type were considered further in an effort to investigate the structure-activity relationships of GPis displaying a 1,4-benzodioxane moiety attached

22, 28, 29

22 → (BF_3•OEt_2, $HSC_2H_4CO_2Me$) R = Ac (**30**), H (**31**) → (a) R = Ac (**32**), H (**33**) → (b) [**34**] → (c) R = Ac (**35**), H (**36**)

predicted K_i = 0.59 μM

Conditions: a: Oxone, NaOAc, AcOH or *m*CPBA, $NaHCO_3$, CH_2Cl_2; b: DBU, $CHCl_3$; c: H_2NOSO_3H, water.

Scheme 5. *S*-β-D-Glucopyranosides and *S*-β-D-glucopyranosyl sulfonamide as GPis.

37

R = Bz (**38**), H (**39**)

R = Bz (**42**), H (**43**)

R = Bz (**40**), H (**41**)
R' = C_6H_5 (**a**), 2-pyridyl (**b**), benzothiazolyl (**c**)

Conditions: a: EtOH, Ag_2CO_3, CH_2Cl_2; b: R'SH, K_2CO_3, acetone; c: $PhNH_2$, CH_2Cl_2.

Scheme 6. *O*-, *S*-, and *N*-Glycosides of hept-2-ulosopyranosonamides as GPis (β-D-*gluco*).

to the aglycon part (Scheme **7**) [133]. Upon treatment with trimethylphosphine, the β azide **44** evolved to an intermediate phosphinimine that was acylated with various carboxylic acids to afford **45d-i**, then the deacetylated products **46d-i** [134]. Their inhibiting properties, compared to that of previously prepared analogs **46a-c** were found at best in the range of that recorded for benzamide **46a**, and lower to that of the 2-naphthyl derivative **46c**, despite structural similarity. It can be seen from the measured K_i / IC_{50} values that the contribution of the 1,4-benzodioxane residue was less detrimental when the nonplanar heterocyclic moiety corresponded to the internal ring, independently of its chirality as in **46g** and **46h**, and that increasing the planarity and aromatic character as in **46i** led to the best inhibitor in this series. The results from these β-D-glucopyranosylamide type GPis underline the importance of the large and unperturbed aromatic part in the aglycon, and indicate that, as in other series, the 2-naphthyl residue is the best choice as yet.

In continuation of previous studies [103, 104], syntheses were carried out to prepare β-configured D-glucopyranosyl-ureas, and D-glucopyranosyl-biurets (Scheme **8**) [135]. To this end, azide **44** was converted under Pintér's conditions [136] to D-glucopyranosyl-urea **47** that was acylated with acyl chlorides to afford products **48a-c**, also accessible by catalytic reduction of **44** to amine **49** followed by treatment with appropriate aroylisocyanates [137]. In a related approach, reaction of amine **49** with arylisocyanates led to *N*-substituted-D-glucopyranosyl-ureas **50a-c**. Amine **49** was converted to β-D-glucopyranosyl-isocyanate **51** by use of $(Cl_3CO)_2CO$, and their condensation led to bis-glucopyranosylurea **50d**. Reaction of isocyanate **51** with phenylurea led to biuret **52a** in moderate yield, while symmetrical analog **52d** was obtained from **49** by reaction with the bielectrophilic reagent chloroformylisocyanate OCNCOCl using 0.5 eq at room temperature. When the temperature was lowered to -26°C, the acylations proceeded stepwise in one pot by the reaction of **49** with OCNCOCl 1 eq, and subsequent addition of different glycosylamines (D-*xylo*, D-*galacto*) to afford **52e,f** in good yield. Upon deacetylation of ureas **50a-d**, acylureas **48a-c** and biurets **52a,d-f**, the corresponding new compounds were evaluated as GPis, for comparison with previously reported data [103, 104]. Structures displaying two hydrophilic sugar moieties (**53d**, **54d-f**) were found weak inhibitors of RMGP*b*. This study indicated that molecules with a glucopyranosyl ring linked to an aromatic moiety *via* a carbonylurea are best inhibitors, compared to analogs linked by urea or biuret linkages. Earlier studies have shown that aryl groups are generally beneficial to the binding, as already discussed [104]. This was particularly clear when comparing the K_i values of **6a** (4.6 μM) to that of *N*-acetyl-β-D-glucopyranosyl-urea **5** (305 μM) [116]. Introduction of a 2-naphthyl group led to the more efficient inhibitor (K_i = 0.4 [137], 0.35 [104] μM/RMGP*b*). Having in mind the structural informations and inhibitions observed with the 1,4-benzodioxane series, one can assume that the planar 2-naphthyl residue in molecule **6c** can bind tightly to the β-channel without disturbing the binding of the D-glucopyranosyl unit. Crystallographic analyses and other data to be reported in full shortly, will explain in detail the binding mode to the active site of GP, and/or possibly to other ones such as the new allosteric site [116] and the newly discovered site [77]. Selected structural information about the complex RMGP*b*-**6c** and related ones appeared when the interactions network in the complexes depending on the ligand's structure was discussed in relation with the kinetic data collected [104]. While H-bonding between the NH group and the CO of His377 (typically 2.9 to 3.3 Å) was generally observed for *N*-acyl-β-D-glucopyranosylamines and *N*-substituted-*N*'-β-D-glucopyranosylureas, the strong affinity of 2-naphthoyl-urea **6c** for RMGP*b* was rather attributed to its extensive interactions with the protein.

d. *N*-β-D-Glucopyranosyl pyrimidines as GPis

A previous study [104] concluded that β-D-glucopyranosyl nucleosides built with uracyl (**55a**), 5-methyl-uracyl (**56a**, R' = Me), and cytosine (**57a**) are good inhibitors of RMGP*b* with K_i values equal to 6.1, 6.6, and 7.7 μM, respectively, compared to analogs synthesized from adenine (K_i = 315 μM) or cyanuric acid (K_i = 1260 μM). Results for analogs in which the 3-OH group in the D-glucopyranosyl ring (Scheme **9**) has been replaced by an

AcO, AcO, AcO, AcO, N_3 **44** — 1 - PMe_3; 2 - RCO_2H → RO, RO, RO, RO, NHCOR; R = Ac (**45a-i**), H (**46a-i**)

Scheme 7. *N*-β-D-Glucopyranosylamides as GPis.

Inhibitory Activity Against RMGP*b*

		K_i (μM)	IC_{50} (μM)
46a	phenyl	81, 144 (a)	-
46b	1-naphthyl	444	-
46c	2-naphthyl	10	-
46d		252±7	630±35
46e	OMe	232±6	580±25
46f	Br	268±7	670±33
46g	H	128±5	320±15
46h	H	120±7	300±15
46i		85±5	125±12

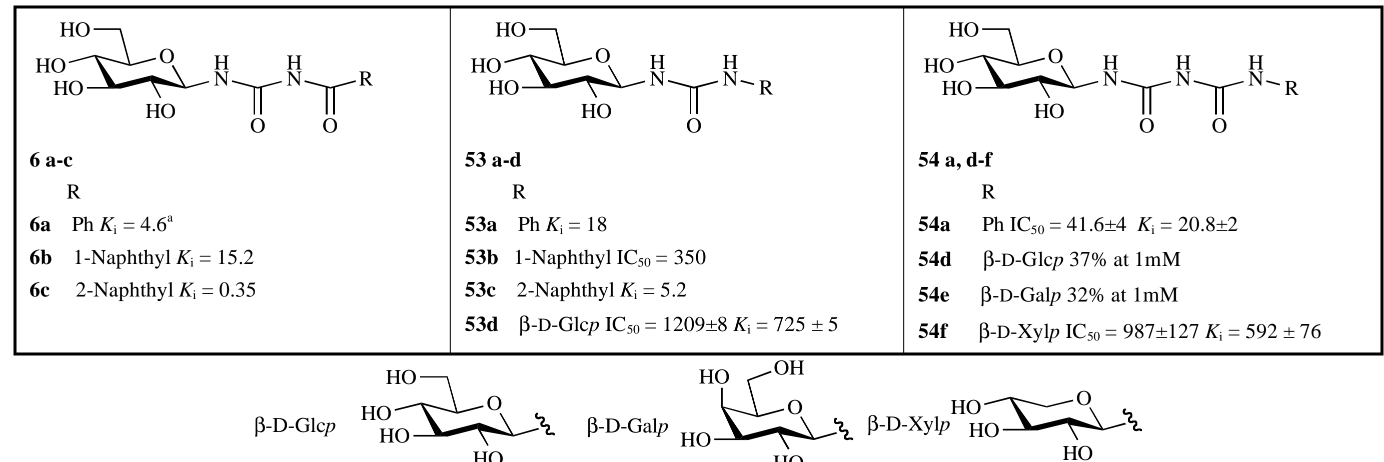
(a): 2 independent measurements for **46a**. see [134] for details.

equatorially oriented fluorine atom have been published lately [115]. As was anticipated from the decreased inhibitions measured when the hydroxyl groups at the 3, 4, or 6 position were replaced by a fluorine atom in D-glucopyranose taken as reference, the inhibitions measured for the fluoro compounds **55b**, **56b** R' = F, and **57b** against RMGP*b* were approximately 500 fold lower, compared to those of **55a**, **56a**, and **57a**. This certainly resulted from the modified glucose ring, as the bifluoro compound **56b** had inhibition potency similar to that of monofluoride **55b**. Interestingly, *N*-benzoylation of the cytosine residue had a positive impact, resulting in a 100 fold enhanced inhibition of **58** (K_i = 46.42 μM), but the absence of the 2-OH group in the 2-deoxy-glucose analogue **59** (K_i = 6.55 mM) led to a 140 fold decreased inhibition compared to **58**. The binding of compounds **55b**, **56b**, **57b**, **58**, and **59** to the catalytic site (whereby the less active T state of the enzyme was promoted, due to several favorable contact with residues of the 280s loop) has been reported, and compared to that of *N*-acetyl-β-D-glucopyranosylamine **2**. In **55b**, **56b**, **57b**, and **58**, the mode of binding and the interactions that the D-glucopyranosyl ring make with GP residues are almost identical with those for α-D-glucose: the 3-OH group of α-D-glucose and the fluorine atom in the five compounds studied formed hydrogen bonds with the main chain amide of Ala673, Ser674, and Gly675. For **2**, similar interactions with the enzyme were observed except that with Ala673. However, comparing the kinetic data for **55** and **57** (**a** versus **b**) showed the fluorine substitution has a profound effect on the potency of the ligands, probably for two reasons. First, for **55a** and **57a**, the 3-OH group formed a hydrogen bond with the side chain of Glu672 (not formed with **55b** and **57b** as the fluorine cannot act as donor in hydrogen bond interactions) [104]. Second, the greater electronegativity and lower polarisability of fluorine over oxygen modify its electrostatic influence and render it a poorer hydrogen bond acceptor. For the five compounds tested, the pyrimidine groups were located between residues 284-286 of the 280s loop, Ala383 of the 380s loop, and His341 of the *β*-pocket near the catalytic site, with stabilization of the closed conformation of the 280s loop. For **56b**, the pyrimidine ring was slightly tilted with respect to its position in **55b** and **57b**. The higher affinity of **58** for the enzyme can be attributed to the hydrogen bonds and van der Waals interactions of the N^4-benzoyl cytosine group with the protein at the *β*-pocket. In **58** and **59**, the benzoyl moiety was accommodated differently, and the rotation by 60° in **59** compared to **58** might have energy cost, which, cumulated with the modifications at the 2 and 3 positions, can explain the lower affinity of compound **59**. Tautomeric forms of the 5 ligands studied were considered as potential binding states and, using Glide-XP docking and QM/MM calculations, the ligands **58** and **59** were predicted to bind in different tautomeric states in their respective GP*b* complexes. Also, a series of substitutions for the equatorial 3-OH group in the D-glucose ring were envisaged for their potential to improve the binding affinity of glucose-based GP*b* catalytic site inhibitors. Glide-XP and quantum mechanics polarized ligand (QPLD-SP/XP) docking calculations revealed favorable binding at this position to be dominated by hydrogen bond contributions. None of the substitutions (including fluorine) out-performed the native OH substituent which can act both as hydrogen bond donor and acceptor, but the NH_2 group and Br atom are the best performers as OH substitutes [115].

e. *C*-β-D-Glucopyranosyl-oxadiazole and 1-(β-D-glucopyranosyl)-1,2,3-triazole Derivatives as GPis

Three series of isomeric D-glucopyranosyl-oxadiazole derivatives have been prepared from a single precursor **19**

Inhibition (%, K_i or IC_{50}) against RMGP*b* (μM)



6 a-c	**53 a-d**	**54 a, d-f**
R	R	R
6a Ph $K_i = 4.6$[a]	**53a** Ph $K_i = 18$	**54a** Ph $IC_{50} = 41.6 \pm 4$ $K_i = 20.8 \pm 2$
6b 1-Naphthyl $K_i = 15.2$	**53b** 1-Naphthyl $IC_{50} = 350$	**54d** β-D-Glc*p* 37% at 1mM
6c 2-Naphthyl $K_i = 0.35$	**53c** 2-Naphthyl $K_i = 5.2$	**54e** β-D-Gal*p* 32% at 1mM
	53d β-D-Glc*p* $IC_{50} = 1209 \pm 8$ $K_i = 725 \pm 5$	**54f** β-D-Xyl*p* $IC_{50} = 987 \pm 127$ $K_i = 592 \pm 76$

[a]a similar data ($K_i = 5.6$ μM) has been reported [116].

Scheme 8. *N*-β-D-Glucopyranosyl(acyl)ureas and biurets as GPis.

(Scheme **10**). The 1,3,4-oxadiazoles were synthesized through the tetrazole **60** by condensation with activated carboxylic acids to afford the benzoylated derivatives **61a-f** followed by saponification of the benzoate esters to provide the *C*-glycosylated 1,3,4-oxadiazoles **62a-f** [138]. Two regioisomeric 1,2,4-oxadiazoles were synthesized displaying the *C*-glucosyl moiety at the 5-position and an aromatic residue at the 3-position of the heteroaromatic ring (**64a-f**), or vice-versa for derivatives **67a-f**. 1,3-Dipolar cycloaddition of nitrile oxides to the D-glucosyl cyanide **19** afforded the benzoylated intermediates **63a-f** and subsequent deprotection afforded the desired oxadiazoles **64a-f** [138, 139]. Also prepared from **19**, amidoxime **65** was subjected to *O*-acylation with various acyl chlorides and cyclo-dehydration to afford the oxadiazoles **66a-f** which were then deprotected to the corresponding hydroxylated derivatives **67a-f** [138, 140].

N-Glucopyranosyl 1,2,3-triazole derivatives were prepared from β-D-glucopyranosyl azide **44** through copper-catalyzed 1,3-dipolar cycloaddition with alkynes [141]. The acetylated intermediates **68a-h** were then deprotected to the corresponding hydroxylated *N*-glucopyranosyl 1,2,3-triazoles **69a-h** (Scheme **11**). α-Configured epimers and β-configured analogs having an axial $CONH_2$ group attached to the anomeric position were also prepared [142].

The study of the inhibition of RMGP*b* by the synthesized glucose-based heterocycles revealed varying potencies depending primarily on the isomeric families considered (Table **2**). While the 1,3,4-oxadiazoles **62a-f** displayed practically

	R	K_i
55a	OH	6.1 µM
55b	F	3.46 mM

	R	R'	K_i
56a	OH	Me	6.6 µM
56b	F	F	3.67 mM

	R	K_i
57a	OH	7.7 µM
57b	F	4.01 mM

58 K_i : 46.42 µM

59 K_i : 6.55 mM

Scheme 9. 3-Deoxy-3-fluoro-β-D-glucopyranosyl-pyrimidine derivatives as GPis.

60 **61a-f** **62a-f** **19** **63a-f** **64a-f** **65** **66a-f** **67a-f**

Conditions: a: NH_4N_3, DMF; b: RCOCl or RCO_2H/DCC, toluene, reflux; c: NaOMe, MeOH; d: RC(Cl)=NOH, Et_3N, toluene, reflux; e: NH_2OH/HCl, pyridine; f: RCOCl or RCO_2H/EDCI/HOBt, 1,4-dioxane

Scheme 10. *C*-β-D-Glucopyranosylated oxadiazoles as GPis.

44 **68a-h** **69a-h**

Conditions: a: $CuSO_4$, L-ascorbic acid, H_2O, 70°C; b: NaOMe, MeOH or NaOH, MeOH or NH_3, dry MeOH

Scheme 11. 1-(β-D-Glucopyranosyl)-1,2,3-triazoles as GPis.

Table 2. β-D-Glucopyranosyl-Oxadiazoles and Triazoles as Inhibitors of GP (IC_{50} or K_i, μM)

	R			R	
62a	*p*-PhNO$_2$	IC_{50}> 625	**64a**	*p*-PhNO$_2$	IC_{50}= 650
62b	Ph	IC_{50}> 625	**64b**	Ph	K_i = 64
62c	*p*-PhMe	IC_{50}> 625	**64c**	*p*-PhMe	K_i = 8.8
62d	*p*-PhOMe	IC_{50}> 625	**64d**	*p*-PhOMe	K_i = 20.4
62e	1-Naphthyl	IC_{50}> 625	**64e**	1-Naphthyl	K_i = 19
62f	2-Naphthyl	IC_{50}> 625	**64f**	2-Naphthyl	K_i = 11.6
	R			R	
67a	*p*-PhNO$_2$	IC_{50}> 625	**69a**	CH$_2$Ph	IC_{50}= 600
67b	Ph	IC_{50}> 625	**69b**	Ph	K_i = 162
67c	*p*-PhMe	IC_{50}= 350	**69c**	CO$_2$Na	IC_{50}> 625
67d	*p*-PhOMe	IC_{50}= 550	**69d**	CO$_2$Me	IC_{50}= 500
67e	1-Naphthyl	IC_{50}> 625	**69e**	1-Naphthyl	IC_{50}= 625
67f	2-Naphthyl	K_i = 38	**69f**	2-Naphthyl	K_i = 36
	For oxadiazoles, see refs [138-140]		**69g**	CONH$_2$	IC_{50}= 350
	For triazoles, see ref [142, 145]		**69h**	CH$_2$OH	K_i = 26

no inhibition of the enzyme (in contrast to an analog with a methyl group as the 5-substituent, K_i = 145 μM [77]), the 1,2,4-oxadiazoles were more potent inhibitors of RMGP*b*. The regioisomery proved to be relevant in this series since the 3-*C*-glucosylated 1,2,4-oxadiazoles **67a-f** displayed much higher K_i values than their regioisomeric counterparts **64a-f**. In addition, the best aromatic group in terms of inhibition is always the 2-naphthyl residue which presumably creates favorable interactions with the catalytic site of the enzyme by entering into the β channel.

The α-configured triazoles α-**69b** and α-**69g** did not display any inhibition towards RMGP*b* (IC_{50} > 625 μM), and α-**69c** (IC_{50} = 400 μM), α-**69d** (30 % inh. at 625 μM), and α-**69h** (IC_{50} = 670 μM) were weak inhibitors. However, the β-configured triazoles **69a-h** inhibited RMGP*b* with K_i values in the μmolar range and again the 2-naphthyl residue was present in the most potent member in the family. In addition, the hydroxymethylated 1,2,3-triazole **69h** displayed a strong inhibition for RMGP*b* and appears as an interesting member of this family [142].

A multivalent system was also studied [143] using oxadiazoles as the binding moiety targeting the catalytic site of GP. Even though the inhibition observed were not as high as expected, the multivalency improved slightly the inhibition observed in comparison to the corresponding monovalent oxadiazole synthesized. In a similar approach [144], homo- and heterobivalent inhibitors of GP were designed incorporating D-glucose and pentacyclic triterpene moieties. These molecules displayed inhibition in the low micromolar range.

f. D-Glucopyranosylidene-spiro-isoxazoline and -spiro-1,4,2-oxathiazole Derivatives as GPis

Since spiro-bicyclic glucose-based molecules (**10-12**) have been identified as potent inhibitors of GP, the design of spiro-isoxazolines **72a-d,f** and spiro-oxathiazoles **76a-g** appeared as a promising approach for the inhibition of GP.

The synthesis of glucose-based spiro-isoxazolines was readily achieved from the methylene *exo*-glycal **70** obtained from the corresponding lactone through a modified Julia olefination [146, 147] and subsequent 1,3-dipolar cycloaddition with nitrile oxides to afford the acetylated **71a-d,f** (Scheme **12**) [139, 148]. The cycloaddition key reaction was found to proceed with high selectivity to afford compounds **71a-d,f** in high yield (83 - 95 % range). Deacetylation under Zemplén conditions led to the hydroxylated spiro-isoxazolines **72a-d,f**, in almost quantitative yields.

Other related spiro compounds were prepared by the nucleophilic attack of thiol **73** on nitrile oxides generated in situ from α-chloro-aldoximes and a base, to afford the corresponding thiohydroximates **74a-g**. These compounds were cyclized under photochemical activation in the presence of *N*-bromosuccinimide to obtain the acetylated (**75a-g**) then hydroxylated glucopyranosylidene-spiro-1,4,2-oxathiazoles **76a-g** (Scheme **13**) [149, 150]. The spiro-cyclization occurred with high stereocontrol as observed for radical reactions at the anomeric position [151].

The spiro-isoxazolines **72a-d,f** and spiro-1,4,2-oxathiazoles **76a-g** were proven to be potent inhibitors of RMGP*b* with K_i values in the low micromolar range (Table

70 71a-d,f 72a-d,f

Conditions: a: RC(Cl)=NOH, Et_3N, CH_2Cl_2; b: NaOMe, MeOH

Scheme 12. D-Glucose-based spiro-isoxazolines as GPis.

73 74a-g 75a-g 76a-g

Conditions: a: RC(Cl)=NOH, Et_3N, CH_2Cl_2; b: NBS, hν, CCl_4; c: NaOMe, MeOH

Scheme 13. D-Glucopyranosylidene-spiro-1,4,2-oxathiazoles as GPis.

3), and even the sub-micromolar range for **72f** and **76f** with a 2-naphthyl residue. Both families are therefore among the most promising series of molecules as glucose-based GP inhibitors [139, 148-150].

X-ray crystallographic studies were performed from co-crystals of spiro-isoxazolines **72a-d,f** and RMGP*b* [148]. The binding to the enzyme is conserved for all five spiro-isoxazolines with a stabilization of the less active T-state conformation of GP. In comparison to the binding mode of glucose, several atomic positions were shifted towards the β-pocket for the endocyclic oxygen, anomeric carbon and carbon at the 6-position of the glucose residue. This shift in atomic positions can explain the better interaction of the inhibitors with the enzyme's catalytic site through additional interactions in the β-channel. These inhibitors are creating minor movements of the side chains of residues 282–287 of the 280s loop. The spiro-isoxazoline five-membered ring is slightly distorted with an angle of ~15°. The 2-naphthyl spiro-isoxazoline **72f** displays contacts with main-chain and side chain amino-acid residues of the 280s loop (Asn282, Asp283, Asn284, Phe285) thus holding the 280s loop in its closed T-state conformation. Overall, compound **72f** makes a total of 15 hydrogen bonds and 111 van der Waals interactions of which 40 are contacts to the 2-naphthyl group. A major contribution to the maximal binding affinity observed for **72f** therefore results from the extended interactions of the 2-naphthyl group with protein atoms and the water hydrogen-bonding network in the vicinity of the β-pocket.

Docking calculations were also performed on GP in complex with spiro-isoxazolines **72a-d,f** with constraints on the glucopyranose position. The results reproduced the complex previously analyzed by crystallography and was in agreement with the best ligands identified from docking calculations. Similar investigations of the enzyme-inhibitor complexes with the spiro-oxathiazoles **76a-g** are underway.

g. Iminosugars as GPis

Sugar analogs in which the heterocyclic oxygen atom has been replaced by a nitrogen atom are known as iminosugars or azasugars, a class of both natural and synthetic molecules which have attracted wide attention as inhibitors of glycosidases [152] with high pharmacological potential [153, 154], so that representatives are commonly marketed, as Miglitol **96** (for T2DM) and Zavesca, the *N*-butyl derivative of de-

Table 3. Spiro-Isoxazolines and Spiro-1,4,2-oxathiazoles as Inhibitors of GP (IC_{50} or K_i, µM)

	R		R
72a	*p*-$PhNO_2$ K_i = 92.5	**76a**	*p*-$PhNO_2$ IC_{50} = 250
72b	Ph K_i = 19.6	**76b**	Ph K_i = 26
72c	*p*-PhMe K_i = 7.9	**76c**	*p*-PhCN IC_{50} = 700
72d	*p*-PhOMe K_i = 6.6	**76d**	*p*-PhOMe K_i = 8.2
72f	2-Naphthyl K_i = 0.63	**76e**	*p*-PhPh IC_{50} = 250
	For spiro-isoxazolines, see refs [139, 148]	**76f**	2-Naphthyl K_i = 0.16
	For spiro-oxathiazoles, see refs [149, 150]	**76g**	*p*-PhF K_i = 48

oxynojirimycin (for Gaucher's and other hereditary diseases; see [155] and ref. therein). There are other potential developments, as antiviral agents, male contraceptives, appetite suppressants (for more details see refs [156, 157], and as chemical chaperone see [156] and ref. therein).

Glycogen degradation is catalyzed by two enzymes, GP and debranching enzyme. The debranching enzyme possesses both activities of 4-α-glucanotransferase and amylo-1,6-glucosidase. GP catalyzes the sequential phosphorolysis of α-1,4-linked glucose units until four glucosyl units remain before an α-1,6 branch point. The 4-α-glucanotransferase activity of the debranching enzyme removes a maltotriosyl unit from the α-1,6 branch and attaches it through an α-1,4 glycosidic bond to the free C-4 of the main chain. This allows the continued release of Glc-1-P by GP. The single remaining α-1,6-linked glucosyl unit is then removed as free glucose by the amylo-1,6-glucosidase activity of the debranching enzyme [158]. This is why there is a continuing interest in the synthesis and evaluation of new iminosugars as inhibitors of glycosidases and other enzymes, in particular those related to the glycogen metabolism.

In this context, DAB **13** and a series of 5-membered analogs, either isolated from plants, or synthesized by known procedures were tested as inhibitors of GP*b* (RMGP*b*), amylo-1,6-glucosidase (amylo-1,6-Glu), various α-glucosidases, and few β-glucosidases. Table **4** shows the inhibitions measured with GP*b* and amylo-1,6-Glu, indicating that **13** was the best inhibitor in the series against GP*b* and the de-branching enzyme amylo-1,6-Glu. These combined inhibitions provided an explanation for the anti-hyperglycemic effect in ob/ob mice reported for DAB **13**. All modifications which preserved the structure of DAB **13**, as for **77**, **79**, **80**, **85**, **86**, **87**, **89**, and **90**, resulted in de-

Table 4. 5-Membered Azasugars as Inhibitors of RMGP*b* and Amylo-1,6-glucosidase (*italicized*)[a]

R= H CH$_2$CH$_2$OH **D-DAB 13** ***N*-EtOH-D-DAB 77** IC$_{50}$: 0.43 IC$_{50}$: 100 IC$_{50}$: *8.4* IC$_{50}$: *12* [159]	**L-DAB 78** NI[b] IC$_{50}$: *200* [159]	R H **D-**DABNAc **79** NI[c] Bn *N*Bn-DABNAc **80** NI[c] [156]	R H L-DABNAc **81** NI[c] Bn *N*Bn-DABNAc **82** NI[c] [156]
D-DRB 83 IC$_{50}$: 27 *NI[d]* [159]	**D-DIX 84** IC$_{50}$: 100 *NI[d]* [159]	2-deoxy-**D-DAB 85** NI[b] *48* [159]	
D-DIG 86 NI[b] *11* [159]	**D-DMDP 87** NI[b] *9.8* [159] IC$_{50}$: 932 [157]	**L-DMDP 88** NI[b] *not indic.* [159] NI[e] [157]	
homo**DMDP 89** IC$_{50}$: 150 *100* [159]	deoxyhomo**DMDP 90** NI[b] *11* [159]	(3*R*)-3-hydroxy-L-bulgecinine **91** IC$_{50}$: 664 [157]	(3*S*)-3-hydroxy-D-bulgecinine **92** NI[e] [157]

[a]IC$_{50}$ (μM) - NI: no inhibition - not indic.: not indicated; [b]less than 50% inhibition at 400 μM; [c]less than 30% inhibition at 1000 μM; [d]less than 50% inhibition at 1000 μM ; [e]no inhibition at 1000 μM.

creased and even absence of inhibition of GP*b*. Neither the enantiomer L-DAB **78** nor the NHAc-modified epimeric compounds **79**, **80** and **81**, **82** were GPis, although **79** - **82** inhibited various β-*N*-acetyl-D-hexosaminidases (which suggested they may be useful for treating adult Tay-Sachs and Sandhoff diseases), but practically none of the other enzymes tested [156]. Only DAB **13** and 2,5-dideoxy-2,5-imino-D-mannitol **87** were inhibitors of the β-glucosidase of the human lysosome, while **13**, **77**, **78**, **83** - **90** inhibited various α-glucosidase, often in the low μM range [159]. A recent study reported syntheses starting from either L-, or D-glucuronolactones to prepare the 5-membered azasugars **87**, **88** and **91**, **92**, as enantiomeric pairs: **87** inhibited β-glucosidases and β-galactosidases; **88** was a potent and selective inhibitor of α-glucosidases. GP was not inhibited by **88** and **92**, but weak inhibitions were observed for the related compounds **87** and **91** that are close structural analogs of D-DAB (Table **4**).

A series of eleven 6-membered azasugars, including 1-deoxynojirimycin **93**, fagomine **94**, miglitol **96**, isofagomine **98**, voglibose **101**, acarbose **102** (Table **5**) were evaluated against RMGP*b*, amylo-1,6-glucosidase (from rabbit skeletal muscles), rat intestinal α-glucosidases, α-glucosidases (Caco-2 cell lines), and lysosomal β-glucosidases [158]. It was found that **93** and α-homonojirimycin **95a** have inhibitory potential toward human maltase identical to that of voglibose **101**. A chaperoning activity was found for D-isofagomine **98** only. 1-Deoxynojirimycin **93** and α-homonojirimycin **95a** showed no significant activity toward GP but were potent inhibitors of amylo-1,6-glucosidase, with IC_{50} values of 0.16 (0.19 in Table **5**), and 0.11 μM, respectively. Miglitol **96** appeared as a potent inhibitor of amylo-1,6-glucosidase, as seen for the *N*-hydroxyethyl derivative **77** of D-DAB **13**. The inhibiting effects of **13** (on GP) and **93** (on amylo-1,6-glucosidase) toward glucagon-induced glucose production in primary rat hepatocytes was investigated. The inhibition of glycogen breakdown by **93** reached plateau at 100 μM with 25% inhibition. However, D-DAB **13** inhibited hepatic glucose production with an IC_{50} value of about 9 μM and the inhibition of **13** was further enhanced in the presence of **93**. This result suggested that amylo-1,6-glucosidase inhibitors in combination with GPis may lower glucose level in T2DM [158].

Among a series of seven-membered 1-azasugars displaying potent inhibition towards α-, and β-glycosidases and glucosylceramide transferase, compounds **103** and **104** were neither GP nor amyloglucosidase (from *Aspergillus niger*) inhibitors [155]. Crystallographic binding experiments with compounds **103** and **104**, in the presence of phosphate and preformed crystals of GP*b*, showed no binding at the catalytic or any other regulatory binding site of GP. It seems that

Table 5. 6-, and 7-Membered Azasugars as Inhibitors of RMGP*b* and Amylo-1,6-glucosidase (*italicized*)[a]

1-Deoxynojirimycin **93** NI[b] *0.19* [158]	Fagomine **94** 200 *2.1* [158]	R [158] H α-Homonojirimycin **95a** β-D-Glucopyranosyl **95b** **95a**, **95b** : NI[b] **95a**: *0.11*; **95b**: *6.1*	Miglitol **96** NI[b] *0.39* [158]	
1,5-D-DIX **97** NI[b] *NI[c]*	Isofagomine **98** 5.8 *480*	L-Isofagomine **99** NI[b] *NI[c]*	Valiolamine **100** NI[b] *31*	Voglibose **101** NI[b] *70*
Acarbose **102** NI[b] *NI[c]*			**103** NI [155]	**104** NI [155]

[a]IC_{50} (μM) - NI: no inhibition; [b]less than 50% inhibition at 400 μM; [c]less than 50% inhibition at 1000 μM; NI: no inhibition.

Table 6. Salacinol and Related Sulfide and Sulfoniums as Inhibitors of GP*b*, Amylo-1,6-glucosidase (*italicized*), and of Human Maltase Glucoamylase[a]

Salacinol **105**	D-ATA **106**	unsulfonated Salacinol **107**	unsulfonated Kotalanol **108**
NI[b]	NI[b]	NI[b]	not indic.
48	*NI*[c]	*16*	*not indic.*
[159]	[159]	[159]	K_i = 0.03 μM
			[161]

[a]IC_{50} (μM) against GP*b* - NI: no inhibition; [b]less than 50% inhibition at 400 μM; [c]less than 50% inhibition at 1000 μM.

GP*b* – unlike glycosidases which tolerate seven-membered iminosugars – is more specific towards five and six-membered iminosugars.

Related analogues of salacinol, the naturally-occurring representative of a growing class of zwitterionic glycosidase inhibitors, are receiving close attention for both synthetic aspects [160] and potential applications. Salacinol **105**, 1,4-anhydro-4-thio-D-arabinitol **106** - a sulfur analog of **13**, and desulfonated Salacinol **107** do not inhibit GP*b*, although **105** and **107** inhibited amylo-1,6-glucosidase (amylo-1,6-Glu) and other α-glucosidases [159]. De-*O*-sulfonated kotalanol **108** has been recently reported to be the strongest inhibitor (K_i = 0.03 μM) of human maltase glucoamylase (MGA), being a ~2000-fold stronger inhibitor of this critical intestinal enzyme, compared to Acarbose **102** (K_i = 62 μM) [161].

CONCLUSION

Among the variety of approaches for pharmacological intervention in T2DM, the inhibition of GP with the aim of reducing glucose hepatic output is a validated and thoroughly investigated strategy. Both the academia and health companies participate in the search of potent inhibitors, that might be suitable for long-term treatment. As several inhibitory sites have been identified for GP, interest focus mainly on structures that can bind at either the catalytic, the allosteric, or the new allosteric sites. Glucose-based motifs and azasugars that bind at the active site constitute the most populated class of GPis. During the last two years, significant progresses have been made, since newly proposed motifs have K_i values in the low micromolar and even sub-micromolar range. Without ignoring previously reported structures, new series based on β-D-glucopyranosyl-pyrimidine, 3-(*C*-β-D-glucopyranosyl)-1,2,4-oxadiazole, D-glucopyranosylidene-spiro-isoxazoline and D-glucopyranosylidene-spiro-oxathiazole motifs appear promising. A representative from this last series, with a 2-naphthyl residue was identified as the most potent GPi to date (K_i = 0.16 μM). While no inhibition was observed for sulfonium analogs, D-DAB remains the best inhibitor among five and six-membered iminosugars that showed inhibitory properties toward GP. A study of glucagon-induced glucose production in primary rat hepatocytes has suggested that amylo-1,6-glucosidase inhibitors in combination with GPis may lower glucose level in T2DM. Considering the limitations found for other potent GPis binding at other sites and the complexity of pharmacological development, the potential of glucose-based GPis is still not established firmly and more tests with cells, tissues, animals are required to better establish the risks and merits of these structures, as antidiabetic drugs. Further studies might also confirm other directions where glucose-based GPis could be useful.

ABBREVIATIONS

ADP = adenosine 5'-diphosphate

AMP = adenosine 5'-monophosphate

ATP = adenosine 5'-triphosphate

cDNA = complementary deoxy nucleic acids

EGP = endogenous glucose production

FMN = flavin mono-nucleotide

FAD = flavin adenine di-nucleotide

Glc-1-P = α-D-glucopyranose-1-phosphate

Glc-6-P = D-glucose-6-phosphate

1-GlcNAc = *N*-acetyl-D-glucopyranosyl amine

GP, GP-s = glycogen phosphorylase(s)

GPis = glycogen phosphorylase inhibitors

GS = glycogen synthase

HGP = hepatic glucose production

HLGP = human liver glycogen phophorylase

HMGP = human muscle glycogen phophorylase

IMP = inosine monophosphate

MalP = maltodextrin phosphorylase

NADH = nicotinamide adenine di-nucleotide

Pi = inorganic phosphate

PLP	=	pyridoxal 5'-phosphate
PP	=	protein phosphatase
PP1	=	protein phosphatase-1
PP1c	=	catalytic subunit of protein phosphatase-1
PTP1B	=	protein thyrosine phosphatase 1B
RMGP	=	rabbit muscle glycogen phophorylase
STZ	=	streptozotocin
T2DM	=	type 2 diabetes mellitus

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