Physiological Control of Liver Glycogen Metabolism: Lessons from Novel Glycogen Phosphorylase Inhibitors

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Abstract: Liver glycogen is synthesized in the postprandial state in response to elevated concentrations of glucose and insulin or by activation of neuroendocrine signals and it is degraded in the postabsorptive state in response to changes in the concentrations of insulin and counter-regulatory hormones. Dysregulation of either glycogen degradation or synthesis through changes in allosteric control or covalent modification of glycogen phosphorylase and glycogen synthase leads to disturbance of blood glucose homeostasis. Liver glycogen phosphorylase has a dual role in the control of glycogen metabolism by regulation of both glycogen degradation and synthesis. The phosphorylated form (GPa) is the active form and determines the rate of degradation of glycogen and it is also a potent allosteric inhibitor of the protein complex, involving the glycogen targeting protein G_L and protein phosphatase-1, which catalyses dephosphorylation (activation) of glycogen synthase. Drug discovery programmes exploring the validity of glycogen phosphorylase as a therapeutic target for type 2 diabetes have generated a wide array of selective phosphorylase ligands that modulate the catalytic activity and / or the phosphorylation state (interconversion of GPa and GPb) as well as the binding of GPa to the allosteric site of G_L. Glycogen phosphorylase inhibitors that act in hepatocytes either exclusively by dephosphorylating GPa (e.g. indole carboxamides) or by allosteric inhibition of GPa (1,4-dideoxy-1,4-D-arabinitol) are very powerful experimental tools to determine the relative roles of covalent modification of glycogen phosphorylase and / or cycling between glycogen synthesis and degradation in the mechanism(s) by which insulin and neurotransmitters regulate hepatic glycogen metabolism.

Keywords: Glycogen, glycogen phosphorylase, inhibitors, hepatocytes, liver.

INTRODUCTION: THE ROLE OF THE LIVER IN TYPE 2 DIABETES

The liver maintains blood glucose homeostasis by uptake of glucose in the postprandial state and conversion to glycogen and triglyceride and by production of glucose in the postabsorptive state by glycogenolysis and gluconeogensis. Thus liver glycogen has a central role in the control of blood glucose homeostasis. After a meal the transition from net glycogen degradation to net synthesis is regulated by the increase in concentrations of glucose and insulin in the portal vein and by neuroendocrine mechanisms activated by the increase in glucose concentration in the portal vein [1]. Defects in the mechanisms by which glucose, insulin or neurotransmitters regulate glycogen synthesis or degradation cause disturbance in blood glucose homeostasis.

Type 2 diabetes is a disorder of blood glucose homeostasis that manifests as a progressive rise in fasting blood glucose and impaired glucose tolerance in the postprandial state. Chronic. elevation in blood glucose if not adequately treated results in damage to blood vessels, kidneys, eyes and nerves with a resultant shortening of life expectancy. The rise in fasting plasma glucose is caused by elevated production of glucose by the liver and the impaired glucose tolerance in the postprandial state is in part due to lack of suppression of hepatic glucose production and decreased hepatic glycogen synthesis [2]. Poor glycaemic control in type 2 diabetes is associated with impaired suppression of hepatic glucose production by high glucose concentration, a condition sometimes described as “decreased glucose effectiveness” [3,4]. Hepatic glycogen phosphorylase which catalyses the first reaction in the degradation of glycogen is considered a potential therapeutic target for type 2 diabetes. Accordingly, Drug Discovery Programmes have generated a wide array of selective ligands of glycogen phosphorylase that modulate the activity of this protein [5-7]. These compounds have proved to be very powerful experimental tools to study the regulation of glycogen metabolism by physiological effectors. This paper reviews recent advances in the control of liver glycogen metabolism and its modulation by novel glycogen phosphorylase inhibitors.

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LIVER GLYCOGEN METABOLISM

Glycogen is a branched polymer of glucosyl residues linked together by α-1,4 bonds along the straight chains and by α-1,6 bonds at the branched points. Degradation of glycogen is catalysed by two enzymes, glycogen phosphorylase which catalyses the phosphorolysis of the α-1,4-bond using inorganic phosphate as substrate and the debranching enzyme which has two catalytic activities, 1,4- α-glucanotransferase and amylo- α -1,6-glucosidase [8]. Synthesis of glycogen likewise involves two enzymes, glycogen synthase which catalyses the synthesis of the α-1,4 bonds and branching enzyme which transfers a glucosyl chain of 6 to 8 units to an adjacent chain to form an α-1,6-branch. The chemical pathway for the synthesis of glycogen from glucose *via* glucose 6-P, glucose 1-P and UDP-glucose, the glucosyl

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donor for glycogen synthase (catalyzed respectively by hexokinase / glucokinase; phosphoglucomutase and UDP-glucose pyrophosphorylase) is similar in liver and extrahepatic tissues [8]. Likewise the degradation of glycogen to glucose 6-P *via* glycogen phosphorylase and phosphoglucomutase is also similar in liver and extrahepatic tissues. However, the liver has distinct mechanisms for regulation of glycogen metabolism, consistent with the unique role of liver glycogen in blood glucose homeostasis. Accordingly there are liver-specific isoforms of the glucose transporter (GLUT2), hexokinase (Glucokinase, Gck), glycogen synthase (LGS), glycogen phosphorylase (PGYL) and also regulatory proteins of glucokinase and protein phosphatase-1 (PP1). In addition the liver expresses glucose 6-phosphatase (G6Pc1) for conversion of glucose 6-P to glucose [9-12].

GLUT2 is the predominant glucose transporter in hepatocytes. It mediates the bidirectional transport of glucose and is involved in glucose uptake and production. It is expressed constitutively in the plasma membrane and because of its high activity it maintains the intracellular glucose concentration in near-equilibrium with the extracellular glucose concentration in the hepatic sinusoids which receive blood from the hepatic artery and the portal vein. Glucokinase (hexokinase IV) catalyses the first reaction in glucose metabolism, the ATP-dependent phosphorylation to glucose to glucose 6-P [9]. It differs from the other hexokinase isoforms in its sigmoidal and low-affinity for glucose and its regulation by a specific glucokinase regulatory protein (GKRP) that further modulates the glucose phosphorylation capacity of the liver cell [13]. Because of the high capacity of the glucose transporter and the low affinity of glucokinase, glucose metabolism in the liver cell responds to a wide range of glucose concentrations as occur in the portal vein in the postprandial state. GKRP functions as an inhibitor of glucokinase that sequesters the enzyme as an inactive pool in the nucleus in the postabsorptive state. The rise in blood glucose concentration in the portal vein in the postprandial state causes the dissociation of glucokinase from GKRP and its translocation to the cytoplasm with a consequent rapid increase in the phosphorylation capacity in the cytoplasm [9]. This is paralleled by a rise in the cytoplasmic concentration of glucose 6-P, which like the rate of glucose phosphorylation is a sigmoidal function of glucose concentration [9]. Glucose 6-P is an important allosteric activator of glycogen synthase [14] and inactivator of glycogen phosphorylase [15,16]. Changes in glucose concentration in the portal vein in the postprandial therefore cause rapid changes in the activities of glycogen synthase and phosphorylase, concomitant with the elevation in glucose 6-P [15-17].

REGULATION OF GLYCOGEN SYNTHASE BY COVALENT MODIFICATION AND BY GLUCOSE 6-P

Glycogen synthase, an oligomer of 85kDa units, is regulated by multisite phosphorylation and by allosteric effectors of which glucose 6-P is the major physiological regulator [14,18-21]. The liver isoform is about 70% identical to the muscle isoform and has 7 phosphorylation sites that are designated "2" and "2a" at the N-terminus and "3a", "3b", "3c", "4" and "5" at the C-terminus [18]. These residues are phosphorylated *in vitro* by various kinases including protein kinase A, phosphorylase kinase, glycogen synthase kinase 3 and AMP-activate protein kinase. Phosphorylation inactivates the enzyme by decreasing its affinity for the substrate UDP-glucose and also the V_{max}. Glucose 6-P functions as an allosteric activator of the phosphorylated forms and even the most phosphorylated forms can be fully activated by saturating concentrations of glucose 6-P [14]. The ability of glucose 6-P to fully activate the phosphorylated forms of the enzyme is the basis for the conventional glycogen synthase assay which measures the ratio of enzyme activity in the absence or presence of saturating concentrations of glucose 6-P. This "activity ratio" is therefore a measure of the phosphorylation state of the enzyme. Dephosphorylation of glycogen synthase is catalysed by protein phosphatase-1 (PP1) in association with glycogen targeting proteins [12,22,23]. Glucose 6-P regulates the activity of glycogen synthase not only by allosteric activation of the phosphorylated enzyme but also by making the phosphorylated protein a better substrate for dephosphorylation by PP1 [14] and by regulating its subcellular location [24]. Several studies have shown that there is a linear relation between the activity ratio of glycogen synthase and the cell content of glucose 6-P [14,21]. This can be explained by glucose 6-P induced dephosphorylation of glycogen synthase [8]. However, a further mechanism by which glucose 6-P regulates glycogen synthase activity is by depleting the phosphorylated form of glycogen phosphorylase [15,16] which is an allosteric inhibitor of the glycogen synthase phosphatase activity of the PP1 complex with the glycogen targeting protein G_L [22] (Fig. **1**).

REGULATION OF GLYCOGEN PHOSPHORYLASE BY COVALENT MODIFICATION AND ALLOSTERIC EFFECTORS

Liver glycogen phosphorylase is a homodimer of 97kDa subunits with about 80% homology with the muscle isoform [25,26]. It is regulated by allosteric effectors and by phosphorylation of a single serine residue at the N-terminus. The phosphorylated and dephosphorylated forms are designated GPa and GPb, respectively. Phosphorylation of GPb to GPa is catalysed by phosphorylase kinase, which is activated by protein kinase A and by elevated cytoplasmic calcium concentration whilst dephosphorylation is catalysed by PP1 in association with glycogen targeting proteins [23]. In liver only GPa is catalytically active [8], and accordingly the interconversion of GPb and GPa by phosphorylation / dephosphorylation is a major determinant of the rate of glycogenolysis. Glycogen phosphorylase exists in two conformational states designated as R- (relaxed)-state or T-(tense)-state, which are intrinsically more active or less active, respectively [25,26] (Fig. **2**). Phosphorylation of the N-terminus and certain allosteric ligands (AMP, inorganic phosphate and glucose 1-P) stabilize the R-state whereas other ligands such as glucose, ATP, purine nucleosides and glucose 6-P stabilize the less active T-state. Allosteric effectors bind to at least 5 distinct binding sites, which comprise: (i) the catalytic site (binds glucose, glucose 1-P and inorganic phosphate): (ii) the purine nucleoside inhibitor or caffeine-binding site located near the active site; (iii) an AMP allosteric site also known as the activator site that also binds ATP, glucose 6-P [27] and the C-terminus of G_L [28]; (iv) a

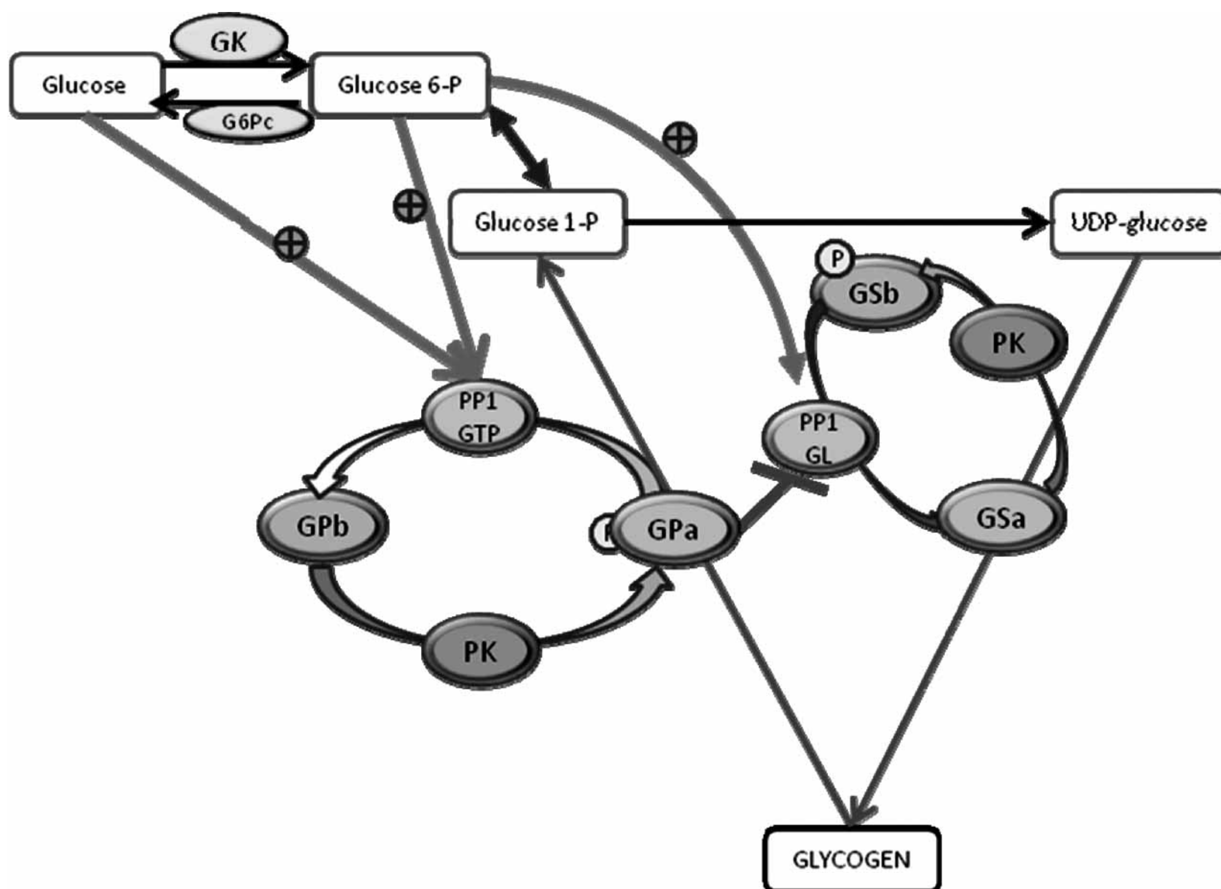


Fig. (1). Dual role of the phosphorylation state of glycogen phosphorylase in regulation of glycogen metabolism.

Glycogen synthesis involves the conversion to glucose to glucose 6-P catalysed by glucokinase and then to glucose 1-P and UDP-glucose, the substrate for glycogen synthase. Glycogen synthase exists as a less active phosphorylated form (GSb) and more active dephosphosphorylated form (GSa). Phosphorylation is catalyzed by multiple protein kinases (PK) and dephosphorylation by PP-1 in association with the glycogen targeting protein GL. Glycogen phosphorylase is converted from an inactive dephosphorylated form (GPb) to an active phosphorylated form (GPa) by phosphorylase kinase (Phk). GPa catalyses the conversion of glycogen to glucose 1-P which is converted to glucose 6-P and then to glucose by glucose 6-phosphatase (G6Pc). Dephosphorylation of GPa is catalysed by protein phosphatase-1 (PP-1) in association with a glycogen targeting protein (GTP). Glucose and glucose 6-P increase the conversion of GPa to GPb by making GPa a better substrate for dephosphorylation by PP-1. Conversion of GSb to GSa is accelerated by glucose 6-P which makes GSb a better substrate for dephosphorylation by PP1-GL and it is inhibited by GPa which is a potent allosteric inhibitor of the glycogen synthase phosphatase activity of PP1-GL.

glycogen storage site; (v) a novel inhibitor site at the subunit interface that binds indole carboxamide ligands [29,30].

Glucose and other ligands that stabilise the T-conformation make liver GPa a better substrate for dephosphorylation by PP1 and thus promote the conversion of GPa to GPb. This is analogous to the action of glucose 6-P on glycogen synthase described above, where glucose 6-P promotes dephosphorylation by PP1-G_L. The action of glucose is synergistic with ligands of the purine nucleoside inhibitor site, which binds various ligands including caffeine and adenosine, which stabilize the T-state [14]. Whether this site has a physiological role in control of hepatic glycogenolysis is still unsettled [31-33]. The AMP activator site is 40 angstrom units from the catalytic site and includes residues from both subunits of the dimer [25,26]. It binds AMP which stabilizes the R-state or ATP and glucose 6-P which stabilize the T-state. Although AMP is a potent activator of muscle GPb increasing activity to 80% of GPa, it has a negligible effect on the activity of liver GPb isoform which remains catalytically inactive in physiological conditions in the hepatocytes [8]. Nonetheless changes in the cellular concentrations of AMP and also glucose 6-P in liver can still have a regulatory role by shifting the equilibrium of GPb between the R-state and T-state and thereby affecting the interconversion of GPa and GPb. Thus incubation of hepatocytes with AICAR (5-aminoimidazole-4-carboxamide 1-beta-D-ribofuranoside) which is metabolized to AICAR-monophosphate a potent AMP analogue, causes conversion of GPb to GPa [16,34]. Conversely metabolic conditions associated with elevated glucose 6-P promote conversion of GPa to GPb [15,16]. The AMP site also binds the C-terminus of the glycogen targeting protein G_L and this mimics AMP binding and stabilises the R-state [28]. Because AMP blocks the binding of G_L to GPa [22], it is predicted to cause activation

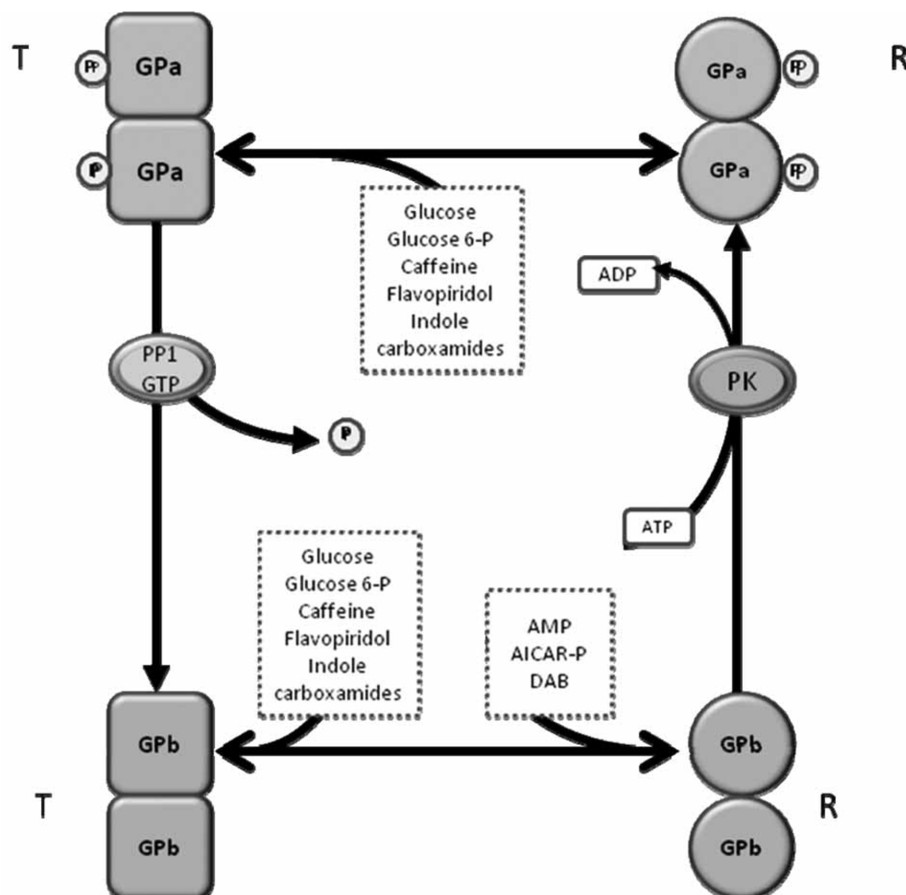


Fig. (2). Glycogen phosphorylase ligands affect the phosphorylation state of glycogen phosphorylase by altering the equilibrium between the relaxed (R) and tense (T) conformations.

Glycogen phosphorylase exists as a homodimer of subunits that have either a relaxed (R) or tense (T) conformation. Certain ligands including glucose, glucose 6-P, caffeine, flavorpiridols and indole carboxamides stabilise the T-conformation, whereas other ligands including AMP, DAB stabilise the R-conformation. The T-conformation of GPa is a better substrate for dephosphorylation by PP-1 whilst the R-conformation is a better substrate for phosphorylation. This model accounts for the conversion of GPa to GPb by glucose, glucose 6-P and certain GPIs and for the conversion of GPb to GPa by DAB and AICAR.

of glycogen synthase by de-inhibition of the synthase phosphatase.

SYNERGISM BETWEEN GLUCOSE AND GLUCOSE 6-P AND RELEVANCE FOR TYPE 2 DIABETES

Conventionally the physiological role of glucose in converting GPa to GPb in liver or isolated hepatocytes has been attributed to a direct effect of glucose in stabilising the T-state [8]. In addition, studies of the inhibition of purified rat liver GPa or recombinant human liver GPa have shown that glucose is a more powerful inhibitor at physiological concentrations than glucose 6-P on GPa activity in solution [35-37]. However, glucose 6-P at physiologically relevant concentrations causes the conversion of GPa to GPb in hepatocytes [15,16]. By using a glucokinase inhibitor to block the conversion of glucose to glucose 6-P, it can be shown that glucose and glucose 6-P act synergistically in promoting conversion of GPa to GPb [16]. In the absence of a glucokinase inhibitor the cellular glucose 6-P concentration is a sigmoidal function of glucose concentration [16], and at a particular glucose concentration it is a linear function of glucokinase activity [9] and inverse function of glucose 6-phosphatase activity [38]. Pharmacological inhibition of glucokinase or overexpression of glucose 6-phosphatase abolishes or suppresses, respectively the elevation in glucose 6-P caused by glucose and suppresses the action of glucose on conversion of GPa to GPb [15,16]. Conversely, elevation of glucose 6-P by glucokinase overexpression enhances the effect of glucose [38]. Thus whilst glucose 6-P is ineffective at inhibiting GPa activity [35-37] it causes conversion of GPa to GPb in the hepatocyte. This is supported by studies using purified GPa demonstrating that glucose 6-P promotes the dephosphorylation of GPa by both PP1 and the PP1- G_L complex [40,41]. The apparent discordance between the efficacy of a ligand at inhibiting GPa in solution and its effect on the interconversion between GPa and GPb in the hepatocyte is evident from the studies with pharmacological GPIs where the effect on GPa to GPb conversion (determined by binding to the T-state) does not necessarily parallel the K_i of the inhibitor on GPa.

Type 2 diabetes is characterized by a decrease in the effectiveness by which high glucose concentration suppresses hepatic glucose production [3,4]. The activity of glucose 6-phosphatase is induced by chronic hyperglycaemia in diabetes [9] and it is elevated in human diabetes [42]. Hepatic glucokinase activity has been reported to be elevated in newly diagnosed type 2 diabetes [43] but decreased in obese subjects with diabetes [44]. High glucose 6-phosphatase expression and / or low glucokinase activity will therefore lead to diminished elevation of glucose 6-P by high glucose and thereby to impaired conversion of GPa to GPb and inappropriate suppression of hepatic glucose production as occurs in diabetes [2-4].

ALLOSTERIC ROLE OF GPa AS INHIBITOR OF GLYCOGEN SYNTHASE PHOSPHATASE

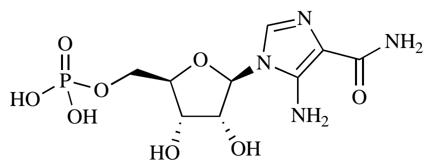
Dephosphorylation of both glycogen phosphorylase and glycogen synthase is catalysed by PP1 in association with glycogen targeting proteins which include G_L, PTG, R6 and RE (encoded respectively by PPP1R3B,C,D,E) [12,22,23]. These proteins each have a PP1-binding domain and a glycogen-binding domain. However, a unique property of G_L, is that it has a high-affinity allosteric binding site for GPa at the C-terminus, designated the GPa-allosteric site [22]. Binding of GPa but not GPb to this allosteric site inhibits glycogen synthase phosphatase activity [45]. This mechanism enables reciprocal regulation of the activation states of glycogen synthase and phosphorylase [8] (Fig. **1**). Although the molecular characterization of the regulation of glycogen synthase phosphatase activity of PP1-G_L by GPa has been elegantly demonstrated *in vitro* [22,45], the physiological role of this mechanism in the glucose-induced activation of glycogen synthase remained debated for many years [8,14]. One hypothesis designated the “sequential mechanism” proposed that conversion of GPa to GPb by glucose is a prerequisite to relieve the inhibition of glycogen synthase phosphatase activity of G_L -PP1 by GPa [8]. Conversely, an alternative hypothesis was that glucose elevates glucose 6-P which directly activates glycogen synthase by dephosphorylation independently of the concentration of GPa [14]. One argument against the sequential mechanism was that various situations are known where glycogen phosphorylase and glycogen synthase are simultaneously active. These include incubation with fructose, adenosine and glutamine, all of which raise the AMP concentration [20]. However, AMP prevents the binding of GPa to G_L [22] providing an explanation for dissociation of the reciprocal coupling of glycogen phosphorylase and synthase in these metabolic conditions. Whether AMP prevents binding by stabilizing the R-conformation or by competing at the same site [28] is not clear, since an AMP-site inhibitor of glycogen phosphorylase (compound A in [48], S. Kauschke, personal communication) was found to have no effect on binding of GPa to G_L [48]. The recent availability of selective ligands of glycogen phosphorylase that stabilise the T-state and convert GPa to GPb [46,47] and of compounds that selectively block the binding of GPa to the C-terminus of G_L [48] has enabled the study of the contribution of the GPa interaction with G_L to the control of glycogen synthase and glycogen synthesis. In addition the generation of an animal model for selective disruption of the allosteric binding site of G_L has confirmed the central role of GPa in regulating the activation state of glycogen synthase [49]. This is unequivocal evidence for the importance of G_L relative to the other glycogen targeting proteins that are expressed in the liver [12] in the regulation of glycogen synthase.

GLYCOGEN PHOSPHORYLASE AS A POTENTIAL THERAPEUTIC TARGET FOR TYPE 2 DIABETES: LESSONS FROM NOVEL GPIs

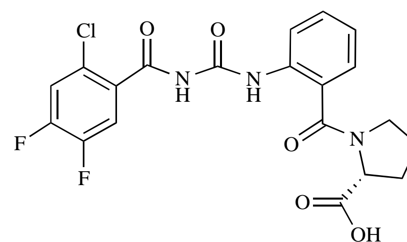
Interest in glycogen phosphorylase as a potential therapeutic target for diabetes initially led to the design and development of glucose analogues that bind to the catalytic site and are more potent inhibitors of glycogen phosphorylase than glucose [50,51]. This can be rationalized from the impaired effectiveness of glucose in suppression of hepatic glucose production in diabetes [2-4]. In the past 15 years Drug Discovery Programmes at major Pharmaceutical Organizations have generated a wide plethora of compounds that bind to glycogen phosphorylase at distinct sites and modulate its catalytic activity and covalent modification *in vivo* [5-7]. Recent developments in this field have been comprehensively reviewed [50,51]. The rest of this paper reviews the information that has emerged from studies on hepatocytes with the novel glycogen phosphorylase inhibitors (GPIs), many of which have been generously provided to the research community by researchers in the Drug Discovery Programmes at Pfizer, Novo Nordisk, Aventis, AstraZeneca, Bayer and others.

LIGANDS OF THE CATALYTIC SITE OF GLYCOGEN PHOSPHORYLASE

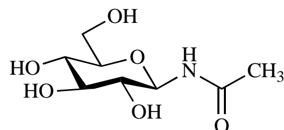
Glucose binds to the catalytic cleft at the glucosyl site that binds glycogen and glucose 1-P and stabilizes the T-conformation. Several glucose analogues have been identified that bind to the glucosyl site with higher affinity than glucose [52-55]. The biological actions of some of these analogues have been studied in hepatocytes. Board and colleagues identified N-acetyl-β-D-glucopyranosylamine (1-GlcNAc) from a systematic analysis of glucose analogue inhibitors of muscle GPb that stabilize the T-state [56,57]. 1-GlcNAc has a K_i on muscle GPb and GPa of 30 μM and 50 μM , respectively as compared with 1.7 and 11 mM for glucose. In liver extracts 1-GlcNAc mimicked the action of glucose in stimulating dephosphorylation of GPa and sequential dephosphorylation of glycogen synthase with a half-maximal effect at less than 1mM compared with ~40-50 mM for glucose. In intact hepatocytes 1-GlcNAc (10 mM) mimicked the action of 50 mM glucose in converting GPa to GPb but unlike glucose did not activate glycogen synthase. The latter was explained by phosphorylation of 1-GlcNAc to 1-GlcNAc-6-P which inhibits the dephosphorylation of glycogen synthase by PP1-G_L irrespective of the presence of glucose 6-P [41]. Interestingly 1-GlcNAc 6-P blocked the dephosphorylation of GPa to GPb by PP1 only in the presence of glucose 6-P (0.5mM or 10 mM). Thus 1-GlcNAc 6-P may compete with glucose 6-P for the nucleotide site on GPa but presumably does not stabilize the T-state. Accordingly the efficacy of 1-GlcNAc in promoting conversion of GPa to GPb in hepatocytes would depend on the relative concentrations of 1-GlcNAc and 1-GlcNAc-6-P [41]. Anderson and Westergaard [58] used 1-GlcNAc as a surrogate for glucose



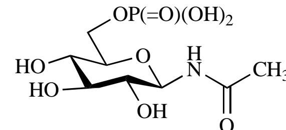
AICAR monophosphate



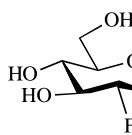
compound A in [48]



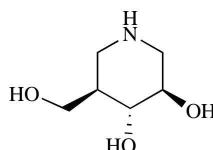
1-GlcNAc



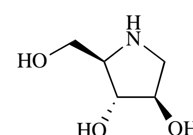
1-GlcNAc 6-P



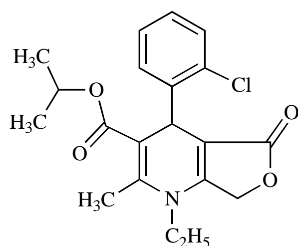
F2-glc



isofagomine



DAB



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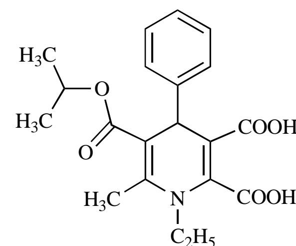

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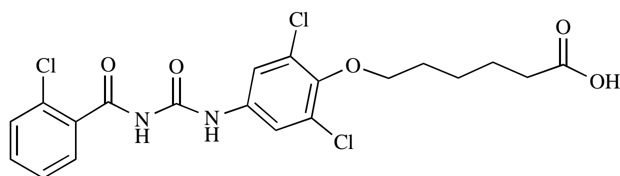
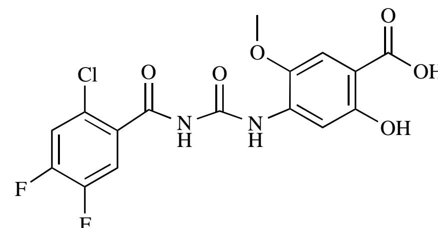


6-{2,6-dichloro-4-[3-(2-chloro-benzoyl)-ureido]-phenoxy}-hexanoic acid



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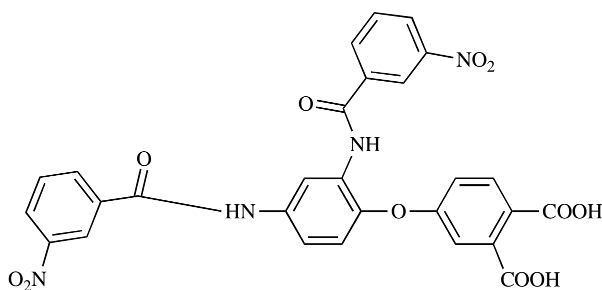
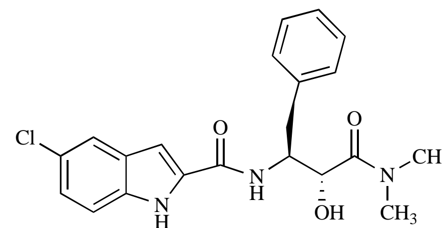


phthalic acid derivative



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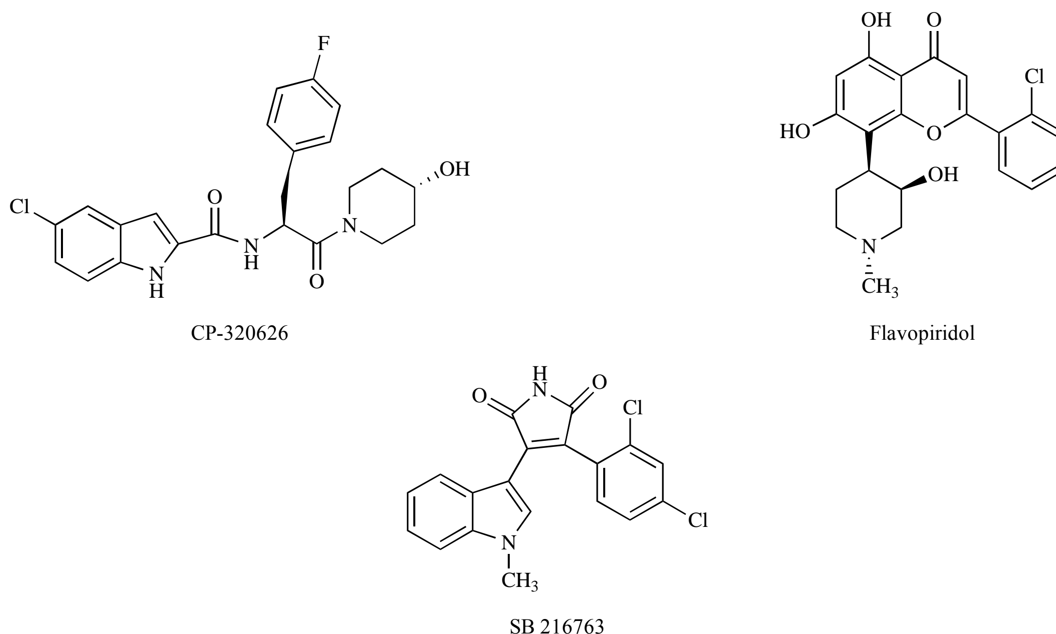

Chart 1. Structure of compounds in the order of appearance in the text.

to study the synergism between "glucose" and other GPIs in assays of glucose production. Interestingly, they found maximum inhibition of glycogenolysis was achieved at 1mM 1-GlcNAc and at this concentration inhibition was only 25%. This contrasts with the near-total (80-90%) inhibition by the other GPIs tested in the same study. The partial (25%) inhibition of glycogenolysis by 1-GlcNAc may be the result of the opposing effects of 1-GlcNAc and 1-GlcNAc 6-P. It is noteworthy that several other glucose analogues were found in our studies (Oikonomakos NG and Agius L, unpublished observations) to be relatively ineffective at inhibition of glycogenolysis in hepatocytes. However, whether this was due to poor cellular uptake or other mechanisms was not determined.

Massillon and colleagues [59] used a glucose analogue that is phosphorylated by hexokinases to a much lesser extent than glucose (2-deoxy-2-fluoro-α-D-glucopyranosyl fluoride, F2-glc) to study the impact of glucose-induced conversion of GPa to GPb on the sequential activation of glycogen synthase in intact hepatocytes. Although 10 mM F2-glc mimicked the action of 50 mM glucose in converting GPa to GPb, it did not cause activation of glycogen synthase (as occurred with 1-GlcNAc). It was inferred that depletion of GPa alone is essential but not sufficient for glucose-induced activation of glycogen synthase and that elevated glucose 6-P is also a pre-requisite [59]. However, more recent studies with indole carboxamides have shown that these compounds cause conversion of GPa to GPb and activation of glycogen synthase and stimulation of glycogen synthesis over a range of glucose concentrations and in the absence of changes in glucose 6-P (see below). Likewise disruption of the G_L-PP1 interaction also causes maximal activation of glycogen synthase irrespective of the glucose concentration [48]. Based on this work with indole carboxamides it can be inferred that other explanations may account for the lack of activation of glycogen synthase by F2-glc [59].

Westergard and colleagues identified the naturally occurring heterocyclic compounds 3-hydroxymethyl 4,5-piperinediol (isofogamine) and 1,4-dideoxy-1,4-D-arabinitol (DAB) as potent inhibitors of glycogen phosphorylase [58,60,61]. Structural studies showed that DAB binds to the active site of muscle GPb and like inorganic phosphate and glucose 1-P stabilizes the R-conformation [62]. In isolated hepatocytes DAB is a very potent inhibitor of both basal and glucagon-stimulated glycogenolysis with half-maximal effect at around 1-2 μM and greater than 80% inhibition of glycogenolysis at 5-20 μM [58,60]. The inhibitory potency was independent of the presence of glucagon and was not associated with net conversion of GPa to GPb [63]. DAB promotes the conversion of GPb to GPa with sequential inactivation of glycogen synthase and inhibition of glycogen synthesis [63]. The potency by which DAB promotes conversion of GPb to GPa is dependent on glucose concentration with half-maximal effect at less than 1 μM at 5mM glucose and at around 10 μM at 25 mM glucose. This can be explained by the opposing effects of glucose and DAB in stabilizing the T-state and R-state, respectively (Fig. **2**). Stabilisation of the R-state by DAB [62] favours the conversion of GPb to GPa, and this in turn inhibits glycogen synthase phosphatase and accordingly glycogen synthesis. Because only GPa is kinetically active in hepatocytes [8], the inhibition of glycogenolysis by DAB can be explained by allosteric inhibition of GPa. To date no glucose analogue ligands of the catalytic site have been shown to cause activation of glycogen synthase or substantial stimulation of glycogen synthesis in hepatocytes or other *in vitro* models.

LIGANDS OF THE AMP ACTIVATOR SITE CAUSE BOTH INACTIVATION AND ALLOSTERIC INHIBITION OF GPa

A dihydropyridine derivative pro-drug (BAY3401) that is metabolized to 1,4-dihydropyridine-2,3-dicarboxylate

(BAY1807) has been studied in perfused liver and isolated hepatocytes [64]. BAY-1807 binds to the AMP-site of muscle GPb and like glucose 6-P stabilizes the T-conformation [65]. The compound caused conversion of GPa to GPb in liver lysates consistent with stabilization of the T-conformation. The prodrug also caused conversion of GPa to GPb in intact hepatocytes but had negligible effect on activation of glycogen synthase when compared with high glucose concentration. Inhibition of glycogenolysis by BAY3401 was attributed to partial dephosphorylation of GPa (conversion to GPb) and to inhibition of the residual GPa by allosteric inhibition [64]. Lack of activation of glycogen synthase was surprising given that the drug stimulated glycogen synthesis *in vivo* [66]. Maslinic acid, a naturally occurring pentacyclic triterpene, is a weak inhibitor of muscle GPa and it binds muscle GPb at the allosteric activator site [67]. Although it caused marginal conversion to GPa to GPb in hepatocytes it did not stimulate glycogen synthesis (Hall, S, Sun H. and Agius, L, unpublished results). Scientists at Aventis identified acylurea derivatives (e.g., 6-{2,6-Dichloro- 4-[3-(2-chloro-benzoyl)-ureido]-phenoxy-hexanoic acid) that are potent inhibitors of liver GPa [68]. Structural studies showed that the compounds bind to muscle GPb at the AMP-activator site and stabilize the T-conformation [69]. Isothermal titration calorimetry showed that the compounds bind far more strongly to GPb (stabilizing the T-state) than to GPa [70]. In hepatocytes S1048, an acylurea derivative caused conversion of GPa to GPb and reciprocal stimulation of glycogen synthesis (Hampson L, Schmoll D, Agius L. unpublished results). Inhibition of glycogenolysis by this acylurea can be at least in part accounted for by conversion of GPa to GPb. However an additional role for allosteric inhibition of GPa as suggested for BAY3401 [64] could not be excluded. Scientists at NovoNordisk identified various phthalic acid derivatives that bind at the AMP-activator site and are potent inhibitors of liver and muscle GPa [71]. These compounds inhibited glycogenolysis in hepatocytes with a greater potency in the absence of glucagon than in its presence (IC_{50} 1.6 vs 4.7 μM). This contrasts with DAB (see above) which inhibits glycogenolysis at similar potency irrespective of the presence of glucagon [60]. Thus compounds that stabilize the R-state and inhibit glycogenolysis exclusively by allosteric inhibition are equally potent regardless of the activation state of phosphorylase kinase. However, compounds that stabilize the T-state and inhibit glycogenolysis at least in part by converting GPa to GPb are less effective when opposed by glucagon.

INDOLE BINDING SITE GPIs INHIBIT GLYCOGENOLYSIS BY CONVERSION OF GPa TO GPb

Researchers at Pfizer identified a series of indole carboxamide derivatives (reviewed in [5]) that bind to a novel site at the subunit interface (2 molecules per homodimer) and stabilize the T-conformation [5,29,46]. CP-91149 inhibits recombinant liver GPa activity synergistically with both glucose or caffeine and it inhibits glucagon-stimulated glycogenolysis in hepatocytes [46]. The synergy with glucose in the kinetic studies is consistent with the structural studies on muscle GPa and GPb demonstrating that glucose and the indole carboxamide (CP-320626) stabilize the T-conformation synergistically [72,73]. In intact hepatocytes CP-91149 promoted the conversion of GPa to GPb and the sequential activation of glycogen synthase [47]. This was associated with very substantial stimulation of glycogen synthesis which correlated inversely with the concentration of GPa [47]. Importantly, the inverse relation between glycogen synthesis and GPa was sustained over a wide range of GPa concentration, indicating that the high control strength of GPa is of physiological significance [47,74]. When the stimulation of glycogen synthesis by the indole carboxamide was expressed as a function of glucose concentration the stimulation manifested as a leftward shift of the glucose-induced stimulation and can be explained by synergy with glucose and glucose 6-P [47,74]. Studies in hepatocytes using another indole carboxamide (CP-320626) also showed marked activation of glycogen synthase and stimulation of glycogen synthesis [75]. There was no increase in glucose 6-P with either compound [39,75] and in some incubation conditions glucose 6-P was decreased by the indole carboxamide [39]. This demonstrates unequivocally that the activation of glycogen synthase by these indolecarboxamides is not due to elevated glucose 6-P. There was also no direct effect of the indole carboxamides on glycogen synthase activity in cell lysates indicating that the activation of glycogen synthase by the compounds in hepatocytes is not due to a direct effect on glycogen synthase. Indole carboxamides cause both conversion of GPa to GPb in hepatocytes [39,47,74] and disruption of the interaction of GPa with the allosteric binding site of G_L [48,76]. The inverse correlation between the activation of glycogen synthase and the concentration of GPa in hepatocytes [63,74] supports depletion of GPa by dephosphorylation as the predominant mechanism that accounts for activation of glycogen synthase.

The effect of low concentrations of CP-91149 on GPa to GPb conversion in hepatocytes was synergistic with glucose and also with glucose 6-P [39] indicating synergy not only between glucose and glucose 6-P in promoting GPa to GPb conversion in hepatocytes [16] but also between indole carboxamides and other T-state ligands. Conversely, the potency of CP-91149 at converting GPa to GPb in hepatocytes was antagonized by AICAR which is metabolized to an AMP analogue [39] and by glucagon [63] which promotes phosphorylation of GPb by activation of phosphorylase kinase, indicating a lower efficacy of the indole carboxamide in metabolic conditions that stabilize the R-state (Fig. **2**). Likewise CP-91149 was a more potent inhibitor of glycogenolysis in the absence of glucagon than in its presence [63], this is similar to the lower potency of AMP-site inhibitors [71], further confirming that compounds that stabilize the T-state are less effective when opposed by activation of phosphorylase kinase.

The hepatocyte studies with CP-91149 show a correlation between glycogenolysis and the cellular content of GPa irrespective of the absence or presence of glucagon [63] suggesting that inhibition of glycogenolysis in the intact cell can be explained by dephosphorylation of GPa. This finding is consistent with the low potency of inhibition of purified liver GPa by indole carboxamides when assayed in a cocktail of physiological regulators (including ATP, ADP, AMP, glucose, glucose 6-P and UDP-glucose) [35]. Cumulatively, these studies support the conclusion that indole carbox-

amides inhibit glycogenolysis in hepatocytes predominantly or exclusively by conversion of GPa to GPb [63]. It can be inferred from the structural studies [29,30,72,73] that since indole carboxamides bind very tightly to the T-conformation but very weakly to the R-state, they shift the allosteric equilibrium towards the T-state and thereby promote conversion of GPa to GPb with negligible allosteric inhibition of GPa. The mechanism of action of indole carboxamides is in marked contrast with the R-state inhibitor, DAB, which inhibits glycogenolysis exclusively by allosteric inhibition of GPa. It can be inferred that other "T-state" inhibitors that show a smaller differential in ligand binding between the T-state and R-state may have dual effects in converting GPa to GPb but also in causing allosteric inhibition of GPa as proposed for the AMP site inhibitor BAY3401 [64].

LIGANDS OF THE PURINE NUCLEOSIDE SITE INHIBIT GLYCOGENOLYSIS IN PART BY ALLOSTERIC INHIBITION

Flavopiridol ((-)-cis-5,7-dihydroxy-2-(2-chlorophenyl)-8-[4-(3-hydroxy-1-methyl)-piperidinyl] -4H-benzopyran-4-one) a naturally occurring inhibitor of cyclin dependent kinases was identified as an inhibitor of muscle GPb and GPa and of phosphorylase kinase induced phosphorylation of GPb [77]. Inhibition of GPb with flavopiridol is synergistic with glucose but additive with inhibition by caffeine [77]. Structural studies showed that flavopiridol binds to muscle GPb at the purine nucleoside site and stabilizes the T-state similar to caffeine and it binds to GPa synergistically with glucose stabilizing the T-state [78]. Flavopiridol (and its 2-phenyl-derivative) suppressed the basal rate of glycogenolysis in hepatocytes but had negligible effect on GPa to GPb conversion indicating that inhibition of basal glycogenolysis is predominantly by allosteric inhibition rather than by dephosphorylation [79]. Consistent with the lack of conversion of GPa to GPb, flavopiridol had little effect on glycogen synthesis. The purine nucleoside site is located on the surface close to the catalytic site [25,26] and allosteric inhibition by flavopiridol (or its derivatives) could presumably be explained by obstruction of the entrance to the catalytic site. Although flavopiridol had little effect on GPa to GPb conversion in basal conditions, it counteracted the conversion of GPb to GPa in hepatocytes by AICAR, which is metabolized to an AMP analogue and by dibutyryl cAMP. The inhibition of glycogenolysis in these conditions could not be fully explained by conversion of GPa to GPb, indicating that it is in part due to allosteric inhibition [79]. Whether the inhibition by flavopiridol and its 2-phenyl derivative of the conversion of GPb to GPa by AICAR and dibutyryl cAMP is in part due to inhibition of phosphorylase kinase [77] could not be ruled out. Studies on ligands of the purine nucleoside site that do not inhibit phosphorylase kinase may help resolve this issue.

INHIBITORS OF THE G_L-GPa COMPLEX

Selective disruption of the binding of GPa to the allosteric inhibitor site at the C-terminus of G_L is a potential strategy to promote glycogen storage by restoring glycogen synthase activity of the G_L-PP1 complex [80,81]. Such a "G_L-GPa" ligand has recently been identified by Zibrova and colleagues and compared in hepatocyte studies with two GPIs, an indole carboxamide and an AMP-site inhibitor. The former but not the latter GPI disrupted binding of GPa to G_L [48]. It is intriguing that whilst G_L binds to the AMP site on GPa, indole carboxamides and AMP but not AMP-site inhibitors block the interaction of GPa and G_L [48]. Both GPIs caused conversion of GPa to GPb and activation of glycogen synthase at both low and high glucose, as shown in other studies with indole carboxamides [47,74,75]. The G_L-GPa ligand did not cause conversion of GPa to GPb, but nonetheless caused activation of glycogen synthase and stimulation of glycogen synthesis [48]. The activation of glycogen synthase was similar by the three compounds confirming that binding of GPa to G_L-PP1 inactivates glycogen synthase. In contrast the stimulation of glycogen synthesis by the G_L-GPa ligand differed from that of the two GPIs both in terms of the maximal effect and time course. The GPIs caused a greater stimulation of glycogen synthesis than the G_L-GPa ligand and whereas in the latter case the stimulation reached a plateau at 2 hours after glucose addition, in the case of the GPIs glycogen storage increased progressively with time after 2 hours. Two explanations are possible for the greater stimulation of glycogen storage by the GPIs than caused by depletion of GPa. First that there is cycling between glycogen synthesis and degradation with the G_L-GPa ligand but not with the GPIs which convert GPa to GPb, as proposed for the murine model of deletion of the allosteric site of G_L [49]. Secondly, that GPa regulates glycogen synthesis by mechanisms additional to allosteric inhibition of G_L or glycogenolytic activity. Other hepatocyte studies [47,82] using an indole carboxamide inhibitor (CP-91149) in conjunction with an inhibitor of GSK-3 (SB216763, arylindolemaleimide) [82] to activate glycogen synthase independently of GPa and in conjunction with DAB to rule out cycling between glycogen synthesis and degradation [83] support the second explanation that depletion of GPa by indole carboxamides stimulates glycogen synthesis to a greater extent than can be explained from activation of glycogen synthase and depletion of the catalytic activity of GPa [47,82]. This indicates a role of GPa in the regulation of glycogen synthesis in hepatocytes by mechanisms additional to: (a) cycling between glycogen synthesis and degradation and (b) inhibition of the synthase phosphatase activity of the G_L-PP1 complex by GPa. Further work with G_L-GPa ligands and indole carboxamides may help identify novel mechanim(s) by which GPa regulates glycogen synthesis.

SUMMARY AND PERSPECTIVES

The above studies on GPIs in hepatocytes show that the mechanism of action of these compounds can be rationalized in terms of a spectrum of mechanisms with DAB and indole carboxamides representing the opposite ends of the spectrum.

a. *DAB*

Structural studies show that DAB stabilizes the R-conformation [62]. In intact hepatocytes DAB promotes conversion of GPb to GPa and it inhibits glycogenolysis exclusively by allosteric inhibition of GPa [63]. Its potency at inhibiting glycogenolysis is independent of the presence of glucagon that promotes conversion of GPb to GPa but it is antagonized by ligands that stabilize the T-state [58]. Because DAB causes conversion of GPb to GPa it also causes

inactivation of glycogen synthase and inhibition of glycogen synthesis at elevated glucose concentration. The potency of DAB on glycogen synthase and glycogen synthesis like its effect on conversion of GPb to GPa is dependent on glucose concentration [63]. Elevated glucose concentration by stabilizing the T-state (synergistically with glucose 6-P) antagonizes the effects of DAB on GPb to GPa conversion.

b. Indole Carboxamides

The action of indole carboxamides on glycogenolysis in hepatocytes can be explained by conversion of GPa to GPb. Structural studies show that indole carboxamides strongly stabilize the T-conformation and bind weakly to the R-state [29,30]. This differential binding to the T and R states may account for the lack of allosteric inhibition of GPa by indole carboxamides in intact hepatocytes. The potency of indole carboxamides at promoting conversion of GPa to GPb (and inhibiting glycogenolysis) is counteracted by phosphorylation and AMP analogues and accordingly potency is decreased in the presence of glucagon. Conversely, glucose 6-P acts synergistically with indole carboxamides in promoting conversion of GPa to GPb and in stabilization of the T-state. Conversion of GPa to GPb in hepatocytes by indole carboxamides is associated with activation of glycogen synthase and stimulation of glycogen synthesis. Activation of glycogen synthase can be explained by reversal of the inhibition of G_L-PPi by GPa. Although indole carboxamides cause disruption of the interaction between GPa and G_L, their action on glycogen synthase activation in the intact cell is most likely fully explained by depletion of GPa. Stimulation of glycogen synthesis by indole carboxamides is greater than can be explained by activation of glycogen synthase alone implicating additional roles of GPa in regulation of glycogen synthesis [47,82]. This may include competition between GPa and glycogen synthase for other glycogen targeting proteins.

Other GPIs that stabilize the T-state but have a smaller differential binding in favour of the T-state relative to the R-state could exert their effects on glycogenolysis in hepatocytes in part by converting GPa to GPb and in part by allosteric inhibition of GPa. Compounds that convert GPa to GPb would have a lower potency on glycogenolysis in the presence of glucagon, as shown for both AMP-site inhibitors and indole carboxamides [60,71]. Conversion of GPa to GPb is expected to be associated with reciprocal activation of glycogen synthase and stimulation of glycogen synthesis as shown by indole carboxamides (and by the G_L-GPa ligand). Lack of activation of glycogen synthase in circumstances associated with conversion of GPa to GPb could be due to non-specific effects of the compound or its metabolites as was shown for 1-GlcNAc [41,56,57]. Several GPIs have been designed and characterized at the molecular level from enzyme kinetics and structural binding studies [50,51]. Few of these compounds have been studied in detail at the cellular level in hepatocytes. Often compounds that are effective inhibitors on the purified enzyme are found to be ineffective at inhibition of glycogenolysis in hepatocytes. This could be due to either low rates of uptake of the compound (which can only be confirmed from uptake studies with the radiolabelled compound) or alternatively to more complex mechanisms as was shown for 1-GlcNAc [41].

In summary, indole carboxamides and DAB when used coordinately are very powerful experimental tools to determine the role of the dephosphorylation state of glycogen phosphorylase in physiological mechanisms such as the mechanism of action of insulin or neurostransmitters on hepatic glycogen metabolism [82,84].

ACKNOWLEDGEMENTS

I am deeply indebted to Dr. Nikos Oikonomakos for many stimulating discussions and collaborations on glycogen phosphorylase. I thank previous colleagues involved in this work, Susan Aiston, Laura Hampson, Theodore Latsis, Andrew Green, Magda Kosmopoulou, Anna-Maria Gomez-Fox, Joan Guinovart, Hongbin Sun. Special thanks are due to Drs Judy Treadway, Birgitte Anderson, Niels Westergaard and Dieter Schmoll for providing the glycogen phosphorylase inhibitors used in our studies. Our research on the physiological regulation of glycogen phosphorylase has been funded by Diabetes UK.

ABBREVIATIONS

AICAR	=	5-aminoimidazole-4-carboxamide 1-beta-D-ribofuranoside
CP-91149	=	R-(R*, S*)]-5-chloro-N-[3-(dimethylamino)-2-hydroxy-3-oxo-1-(phenylmethyl)pr opyl]-1H-indole-2-carboxamide
DAB	=	1,4-dideoxy-1,4-D-arabinitol
F2-glc	=	2-deoxy-2-fluoro-α-D-glucopyranosyl fluoride
1-GlcNAc	=	N-acetyl-β-D-glucopyranosylamine
glucose 1-P	=	glucose 1-phosphate
glucose 6-P	=	glucose 6-phosphate
GPa	=	phosphorylated form of glycogen phosphorylase
GPb	=	unphosphorylated form of glycogen phosphorylase
GPIs	=	glycogen phosphorylase inhibitors
PP1	=	protein phosphatase-1.

REFERENCES

[1] Cherrington, A.D. Banting Lecture 1997. Control of glucose uptake and release by the liver *in vivo*. *Diabetes,* **1999**, *48,*1198-1214.

[2] Basu, A.; Shah P.; Nielsen M.; Basu, R.; Rizza, R.A. Effects of type 2 diabetes on the regulation of hepatic glucose metabolism. *J Investig Med.,* **2004**, 52, 366-374.

[3] Basu, A.; Rizza, R.A. Glucose effectiveness: measurement in diabetic and nondiabetic humans. *Exp Clin Endocrinol Diabetes.* **2001**, *109*(Suppl 2), S157-165.

[4] Hawkins, M.; Gabriely, I.; Wozniak, R.; Reddy, K.; Rossetti, L.; Shamoon, H. Glycemic control determines hepatic and peripheral glucose effectiveness in type 2 diabetic subjects. *Diabetes.,* **2002** *51,* 2179-2189.

[5] Treadway, J.L.; Mendys, P.; Hoover, D.J.Glycogen phosphorylase inhibitors for the treatment of type 2 diabetes mellitus. *Expert Opin Investig Drugs*., **2001**, *10,* 439-454.

[6] Henke, B.R.; Sparks, S.M. Glycogen phosphorylase inhibitors. *Mini Rev. Med. Chem*., **2006**, *6,* 845-857.

[7] Agius, L. New hepatic targets for glycaemic control in diabetes. *Best Pract. Res. Clin. Endocrinol. Metab.,* **2007**, *21*, 587-605/

[8] Bollen, M., Keppens, S.; Stalmans, W. Specific features of glycogen metabolism in the liver. *Biochem. J*., **1998**, *336*, 19-31.

[9] Agius, L. Glucokinase and molecular aspects of liver glycogen metabolism. *Biochem. J*., **2008**, *414,* 1-18.

[10] Van Schaftingen, E.; Gerin, I. The glucose-6-phosphatase system. *Biochem. J*., **2002**, *362,* 513-532.

[11] Newgard, C.B.; Brady ,M.J.; O'Doherty, R.M.; Saltiel, A.R. Organizing glucose disposal: emerging roles of the glycogen targeting subunits of protein phosphatase-1. *Diabetes*, **2000**, *49,* 1967-1977.

[12] Munro, S.; Ceulemans, H.; Bollen, M.; Diplexcito, J.; Cohen. P.T. A novel glycogen-targeting subunit of protein phosphatase 1 that is regulated by insulin and shows differential tissue distribution in humans and rodents. *FEBS J.,* **2005**, *272,* 1478-1489

[13] De la Iglesia, N.; Mukhtar, M.; Seoane, I.; Guinovart, J.J.; Agius, L. The role of the regulatory protein of glucokinase in the glucose sensory mechanism of the hepatocyte. *J. Biol. Chem*., **2000**, *275,* 10597-10603

[14] Ferrer, J.C.; Favre, C.; Gomis, R.R.; Fernández-Novell, J.M.; García-Rocha, M.; de la Iglesia, N.; Cid, E.; Guinovart, J.J. Control of glycogen deposition. *FEBS Lett.,* **2003**, *546,* 127-132

[15] Aiston, S.; Andersen, B.; Agius, L. Glucose 6-phosphate regulates hepatic glycogenolysis through inactivation of phosphorylase. *Diabetes.,* **2003**, *52,* 1333-1339

[16] Aiston, S.; Green, A.; Mukhtar, M.; Agius, L. Glucose 6-phosphate causes translocation of phosphorylase in hepatocytes and inactivates the enzyme synergistically with glucose. *Biochem. J.,* **2004**, *377,* 195-204

[17] Fernández-Novell, J.M.; Roca, A.; Bellido, D.; Vilaró, S.; Guinovart, J.J. Translocation and aggregation of hepatic glycogen synthase during the fasted-to-refed transition in rats. *Eur. J. Biochem*., **1996**, *238,* 570-575.

[18] Ros, S.; García-Rocha, M.; Domínguez, J.; Ferrer, J.C.; Guinovart, J.J. Control of liver glycogen synthase activity and intracellular distribution by phosphorylation. *J. Biol. Chem.,* **2009**, *284,* 6370-6378

[19] Carabaza, A.; Ciudad, C.J.; Baqué, S.; Guinovart, J.J. Glucose has to be phosphorylated to activate glycogen synthase, but not to inactivate glycogen phosphorylase in hepatocytes. *FEBS Lett.,* **1992**, *296,* 211-214.

[20] Carabaza, A.; Ricart, M.D.; Mor, A.; Guinovart, J.J.; Ciudad, C.J. Role of AMP on the activation of glycogen synthase and phosphorylase by adenosine, fructose, and glutamine in rat hepatocytes. *J. Biol. Chem*., **1990**, *265,* 2724-2732.

[21] Ciudad, C.J.; Carabaza, A.; Guinovart, J.J. Glucose 6-phosphate plays a central role in the activation of glycogen synthase by glucose in hepatocytes. *Biochem Biophys Res Commun*., **1986**, *141,* 1195-1200

[22] Doherty, M.J.; Moorhead, G.; Morrice, N.; Cohen, P.; Cohen, P.T. Amino acid sequence and expression of the hepatic glycogen-binding (GL)-subunit of protein phosphatase-1. *FEBS Lett.,* **1995**, *375,* 294-298

[23] Browne, G.J.; Delibegovic, M.; Keppens, S.; Stalmans, W.; Cohen, P.T. The level of the glycogen targetting regulatory subunit R5 of protein phosphatase 1 is decreased in the livers of insulin-dependent diabetic rats and starved rats. *Biochem. J.,* **2001**, *360,* 449-459.

[24] García-Rocha, M.; Roca, A.; De La Iglesia, N.; Baba, O.; Fernández-Novell, J.M.; Ferrer, J.C.; Guinovart, J.J. Intracellular distribution of glycogen synthase and glycogen in primary cultured rat hepatocytes. *Biochem. J.,* **2001**, *357,* 17-24.

[25] Newgard, C.B.; Hwang, P.K.; Fletterick,. R.J. The family of glycogen phosphorylases: structure and function. *Crit. Rev. Biochem. Mol. Biol.,* **1989**, *24,* 69-99.

[26] Johnson, L.N. Glycogen phosphorylase: control by phosphorylation and allosteric effectors. *FASEB J.,* **1992**, *6,* 2274-2282.

[27] Johnson, L.N.; Snape P.; Martin, J.L.; Acharya, K.R.; Barford, D.; Oikonomakos, N.G. Crystallographic binding studies on the allosteric inhibitor glucose-6-phosphate to T state glycogen phosphorylase b. *J. Mol. Biol*., **1993**, *232,* 253-267.

[28] Pautsch, A.; Stadler, N.; Wissdorf, O.; Langkopf, E.; Moreth, W.; Streicher, R. Molecular recognition of the protein phosphatase 1 glycogen targeting subunit by glycogen phosphorylase. *J. Biol. Chem*., **2008**, *283,* 8913-8918.

[29] Rath, V.L., Ammirati, M.; Danley, D.E.; Ekstrom, J.L.; Gibbs, E.M.; Hynes, T.R.; Mathiowetz, A.M.; McPherson, R.K.; Olson, T.V.; Treadway, J.L.; Hoover, D.J. Human liver glycogen phosphorylase inhibitors bind at a new allosteric site. *Chem. Biol*., **2000**, *9*, 677-682.

[30] Oikonomakos, N.G.; Skamnaki, V.T.; Tsitsanou, K.E.; Gavalas, N.G.; Johnson, L.N. A new allosteric site in glycogen phosphorylase b as a target for drug interactions. *Structure,* **2000** *8,* 575-584.

[31] Kasvinsky, P.J.; Shechosky, S.; Fletterick, R.J. Synergistic regulation of phosphorylase a by glucose and caffeine. *J. Biol. Chem*., **1978**, *253,* 9102-9106.

[32] Ekstrom, J.L.; Pauly, T.A.; Carty, M.D.; Soeller, W.C.; Culp, J.; Danley, D.E.; Hoover, D.J.; Treadway, J.L.; Gibbs, E.M.; Fletterick, R.J.; Day, Y.S.; Myszka, D.G.; Rath, V.L. Structure-activity analysis of the purine binding site of human liver glycogen phosphorylase. *Chem. Biol*., **2002**, *9,* 915-924.

[33] Ercan-Fang, N.G.; Nuttall, F.Q.; Gannon, M.C. Uric acid inhibits liver phosphorylase a activity under simulated *in vivo* conditions. *Am. J. Physiol. Endocrinol. Metab*., **2001**, *280,* E248-253.

[34] Shang, J.; Lehrman, M.A. Activation of glycogen phosphorylase with 5-aminoimidazole-4-carboxamide riboside (AICAR). Assessment of glycogen as a precursor of mannosyl residues in glycoconjugates. *J. Biol. Chem*., **2004**, *279,* 12076-80.

[35] Ercan-Fang, N.; Taylor, M.R.; Treadway, J.L.; Levy, C.B.; Genereux, P.E.; Gibbs, E.M.; Rath, V,L.; Kwon, Y.; Gannon, M.C.; Nuttall, F.Q. Endogenous effectors of human liver glycogen phosphorylase modulate effects of indole-site inhibitors. *Am. J. Physiol. Endocrinol. Metab.,* **2005**, *289,* E366-372.

[36] Ercan-Fang, N.; Gannon, M.C.; Rath, V.L.; Treadway, J.L.; Taylor, M.R.; Nuttall, F.Q. Integrated effects of multiple modulators on human liver glycogen phosphorylasen a. *Am. J. Physiol. Endocrinol. Metab.,* **2002**, *283,* E29-37.

[37] Ercan, N.; Gannon, M.C.; Nuttall, F.Q. Allosteric regulation of liver phosphorylase a: revisited under approximated physiological conditions. *Arch. Biochem. Biophys*., **1996**, *328,* 255-264.

[38] Aiston, S.; Trinh, K.; Lange, A.J.; Newgard, C.B.; Agius, L. Glucose 6-phosphatase overexpression lowers glucose 6-phosphate and inhibits glycogen synthesis and glycolysis without affecting glucokinase translocation: evidence against feedback inhibition of glucokinase. *J. Biol. Chem.,* **1999**, *274,* 24559-24566.

[39] Hampson, L.J.; Agius, L. Increased potency and efficacy by combined phosphorylase inactivation and glucokinase activation in the control of hepatocyte glycogen metabolism. *Diabetes,* **2005**, *54,* 617-623

[40] Cadefau, J.; Bollen, M.; Stalmans, W. Glucose-induced glycogenesis in the liver involves the glucose-6-phosphate-dependent dephosphorylation of glycogen synthase. *Biochem. J.,* **1997**, *322,* 745-50.

[41] Board, M. N-Acetyl-beta-D-glucopyranosylamine 6-phosphate is a specific inhibitor of glycogen-bound protein phosphatase 1. *Biochem. J*. **1997**, *328,* 695-700.

[42] Clore, J.N.; Stillman, J.; Sugerman, H. Glucose-6-phosphatase flux *in vitro* is increased in type 2 diabetes. *Diabetes,* **2000**, *49*, 969-974.

[43] Caro, J.F.; Triester, S.; Patel, V.K.; Tapscott, E.B.; Frazier, N.L.; Dohm, G.L. Liver glucokinase: decreased activity in patients with type II diabetes. *Horm. Metab. Res.,***1995**, *27,* 19-22.

[44] Willms, B.; Ben-Ami, P.;Soling, H-D. Hepatic enzyme activities of glycolysis and gluconeogenesis in diabetes of man and laboratory animals. *Horm. Metab. Res.***1970** *2,* 135-141.

[45] Alemany, S.; Cohen, P. Phosphorylase a is an allosteric inhibitor of the glycogen and microsomal forms of rat hepatic protein phosphatase-1. *FEBS Lett.* **1986**, *198*, 194-202.

[46] Martin, W.H.; Hoover, D.J.; Armento, S.J.; Stock, I.A.; McPherson, R.K.; Danley, D.E.; Stevenson, R.W.; Barrett, E.J.; Treadway, J.L. Discovery of a human liver glycogen phosphorylase inhibitor

that lowers blood glucose *in vivo*. *Proc. Natl. Acad. Sci. USA,* **1998**, *95,* 1776-1781.

[47] Aiston, S.; Hampson, L.; Gomez-Foix, A.M.; Guinovart, J.J.; Agius L. Hepatic glycogen synthesis is highly sensitive to phosphorylase activity: Evidence from metabolic control analysis. *J. Biol Chem.,* **2001**, *276,* 23858-23866.

[48] Zibrova, D.; Grempler, R.; Streicher, R.; Kauschke, S.G. Inhibition of the interaction between protein phosphatase 1 glycogen-targeting subunit and glycogen phosphorylase increases glycogen synthesis in primary rat hepatocytes. *Biochem. J.,* **2008**, *412,* 359-366.

[49] Kelsall, I.R.; Rosenzweig, D.; Cohen, P.T. Disruption of the allosteric phosphorylase a regulation of the hepatic glycogen-targeted protein phosphatase 1 improves glucose tolerance *in vivo*. *Cell Signal.,* **2009**, *21,*1123-1134.

[50] Somsák, L.; Czifrák, K.; Tóth, M.; Bokor, E.; Chrysina, E.D.; Alexacou, K.M.; Hayes. J.M.; Tiraidis, C.; Lazoura, E.; Leonidas, D.D.; Zographos, S.E.; Oikonomakos, N.G. New inhibitors of glycogen phosphorylase as potential antidiabetic agents. *Curr. Med. Chem.,* **2008**, *15,* 2933-2983.

[51] Oikonomakos, N.G.; Somsák, L. Advances in glycogen phosphorylase inhibitor design. *Curr. Opin. Investig. Drugs,* **2008**, *9,* 379-395.

[52] Martin, J.L.; Veluraja, K.; Ross, K.; Johnson, L.N.; Fleet, G.W.; Ramsden, N.G.; Bruce, I.; Orchard, M.G.; Oikonomakos, N.G.; Papageorgiou, A.C. Glucose analogue inhibitors of glycogen phosphorylase: the design of potential drugs for diabetes. *Biochemistry,* **1991**, *30,* 10101-10116.

[53] Watson, K.A.; Mitchell, E.P.; Johnson, L.N.; Son, J.C.; Bichard, C.J.; Orchard, M.G.; Fleet, G.W.; Oikonomakos, N.G.; Leonidas, D.D.; Kontou, M. Design of inhibitors of glycogen phosphorylase: a study of alpha- and beta-C-glucosides and 1-thio-beta-D-glucose compounds. *Biochemistry,* **1994**, *33,* 5745-5758.

[54] Oikonomakos, N.G.; Kontou, M.; Zographos, S.E.; Watson, K.A.; Johnson, L.N.; Bichard, C.J.; Fleet, G.W.; Acharya, K.R. N-acetyl-beta-D-glucopyranosylamine: a potent T-state inhibitor of glycogen phosphorylase. A comparison with alpha-D-glucose. *Protein Sci.,* **1995**, *4,* 2469-2477.

[55] Gregoriou, M.; Noble, M.E.; Watson KA, Garman EF, Krulle TM, de la Fuente C, Fleet GW, Oikonomakos NG, Johnson LN. The structure of a glycogen phosphorylase glucopyranose spirohydantoin complex at 1.8 A resolution and 100 K: the role of the water structure and its contribution to binding. *Protein Sci.,* **1998**, *7,* 915-927.

[56] Board, M.; Bollen, M.; Stalmans, W.; Kim, Y.; Fleet, G.W.; Johnson, L.N. Effects of C-1-substituted glucose analogue on the activation states of glycogen synthase and glycogen phosphorylase in rat hepatocytes. *Biochem. J.,* **1995**, *311,* 845-852

[57] Board, M.; Hadwen, M.; Johnson, L.N. Effects of novel analogues of D-glucose on glycogen phosphorylase activities in crude extracts of liver and skeletal muscle. *Eur. J. Biochem.,* **1995**, *228,*753-761.

[58] Andersen, B.; Westergaard, N. The effect of glucose on the potency of two distinct glycogen phosphorylase inhibitors. *Biochem. J.,* **2002**, *367,* 443-50.

[59] Massillon, D.; Bollen, M.; De Wulf, H.; Overloop, K.; Vanstapel, F.; Van Hecke, P.; Stalmans, W. Demonstration of a glycogen/glucose 1-phosphate cycle in hepatocytes from fasted rats. Selective inactivation of phosphorylase by 2-deoxy-2-fluoro-alpha-D-glucopyranosyl fluoride. *J. Biol. Chem.,* **1995**, *270,* 19351-19356

[60] Andersen, B.; Rassov, A.; Westergaard, N.; Lundgren, K. Inhibition of glycogenolysis in primary rat hepatocytes by 1, 4-dideoxy-1,4-imino-D-arabinitol. *Biochem. J.,* **1999**, *342,* 545-550.

[61] Fosgerau, K.; Westergaard, N.; Quistorff, B.; Grunnet, N.; Kristiansen, M.; Lundgren, K. Kinetic and functional characterization of 1,4-dideoxy-1, 4-imino-d-arabinitol: a potent inhibitor of glycogen phosphorylase with anti-hyperglyceamic effect in ob/ob mice. *Arch. Biochem. Biophys.,* **2000**, *380,* 274-284.

[62] Oikonomakos, N.G.; Tiraidis, C.; Leonidas, D.D.; Zographos, S.E.; Kristiansen, M.; Jessen, C.U.; Norskov-Lauritsen, L.; Agius, L. Iminosugars as potential inhibitors of glycogenolysis: structural insights into the molecular basis of glycogen phosphorylase inhibition. *J. Med. Chem.,* **2006** *49,* 5687-5701.

[63] Latsis, T.; Andersen, B.; Agius, L. Diverse effects of two allosteric inhibitors on the phosphorylation state of glycogen phosphorylase in hepatocytes. *Biochem. J.,* **2002**, *368,* 309-316.

[64] Bergans, N.; Stalmans, W.; Goldmann, S.; Vanstapel, F. Molecular mode of inhibition of glycogenolysis in rat liver by the dihydropyridine derivative, BAY R3401: inhibition and inactivation of glycogen phosphorylase by an activated metabolite. *Diabetes,* **2000**, *49,* 1419-1426.

[65] Zographos, S.E.; Oikonomakos, N.G.; Tsitsanou, K.E.; Leonidas, D.D.; Chrysina, E.D.; Skamnaki, V.T.; Bischoff, H.; Goldmann, S.; Watson, K.A.; Johnson, L.N. The structure of glycogen phosphorylase b with an alkyldihydropyridine-dicarboxylic acid compound, a novel and potent inhibitor. *Structure,* **1997**, *5,*1413-1425.

[66] Shiota, M.; Jackson, P.A.; Bischoff, H.; McCaleb, M.; Scott, M.; Monohan, M.; Neal, D.W.; Cherrington, A.D. Inhibition of glycogenolysis enhances gluconeogenic precursor uptake by the liver of conscious dogs. *Am J Physiol.,* **1997**, *273*, E868-879.

[67] Wen, X.; Sun, H.; Liu, J.; Cheng, K.; Zhang, P.; Zhang, L.; Hao, J.; Zhang, L.; Ni, P.; Zographos, S.E.; Leonidas, D.D.;, Alexacou, K.M.; Gimisis, T.; Hayes, J.M.; Oikonomakos, N.G. Naturally occurring pentacyclic triterpenes as inhibitors of glycogen phosphorylase: synthesis, structure-activity relationships, and X-ray crystallographic studies. *J. Med. Chem.,* **2008**, *51*, 3540-3554.

[68] Klabunde, T.; Wendt, K.U.; Kadereit, D.; Brachvogel, V.; Burger, H.J.; Herling, A.W.; Oikonomakos, N.G.; Kosmopoulou, M.N.; Schmoll, D.; Sarubbi, E.; von Roedern, E.; Schönafinger, K.; Defossa, E. Acyl ureas as human liver glycogen phosphorylase inhibitors for the treatment of type 2 diabetes. *J. Med. Chem.,* **2005**, *48,* 6178-93.

[69] Oikonomakos, N.G.; Kosmopoulou, M.N.; Chrysina, E.D.; Leonidas, D.D.; Kostas, I.D.; Wendt, K.U.; Klabunde, T.; Defossa, E. Crystallographic studies on acyl ureas, a new class of glycogen phosphorylase inhibitors, as potential antidiabetic drugs. *Protein Sci.,* **2005**, *14,* 1760-1771.

[70] Anderka, O.; Loenze, P.; Klabunde, T.; Dreyer, M.K.; Defossa, E.; Wendt, K.U.; Schmoll, D. Thermodynamic characterization of allosteric glycogen phosphorylase inhibitors. *Biochemistry,* **2008**, *47,* 4683-4691.

[71] Kristiansen, M.; Andersen, B.; Iversen, L.F.; Westergaard, N. Identification, synthesis, and characterization of new glycogen phosphorylase inhibitors binding to the allosteric AMP site. *J. Med. Chem.,* **2004**, *47,* 3537-3545.

[72] Oikonomakos, N.G.; Zographos, S.E.; Skamnaki, V,T.; Archontis, G. The 1.76 A resolution crystal structure of glycogen phosphorylase B complexed with glucose, and CP320626, a potential antidiabetic drug. *Bioorg. Med. Chem.,* **2002**, *10,* 1313-1319.

[73] Oikonomakos, N.G.; Chrysina, E.D.; Kosmopoulou, M.N.; Leonidas, D.D. Crystal structure of rabbit muscle glycogen phosphorylase a in complex with a potential hypoglycaemic drug at 2.0 A resolution. *Biochim. Biophys. Acta,* **2003**, *1647,* 325-332.

[74] Arden, C.; Green, A.R.; Hampson, L.J.; Aiston, S.; Harndahl, L.; Greenberg, C.C.; Brady, M.J.; Freeman, S.; Poucher, S.M.; Agius, L. Increased sensitivity of glycogen synthesis to phosphorylase-a and impaired expression of the glycogen targeting protein R6 in hepatocytes from insulin resistant Zucker fa/fa rats. *FEBS J.,* **2006**, *273,* 1989-1999.

[75] Gustafson, L.A.; Neeft, M.; Reijngoud, D.J.; Kuipers, F.; Sauerwein, H.P.; Romijn, J.A.; Herling, A.W.; Burger, H.J.; Meijer, A.J. Fatty acid and amino acid modulation of glucose cycling in isolated rat hepatocytes. *Biochem. J.,* **2001**, *358,* 665-671.

[76] Kelsall, I.R.; Munro, S.; Hallyburton, I.; Treadway, J.L.; Cohen, P.T. The hepatic PP1 glycogen-targeting subunit interaction with phosphorylase a can be blocked by C-terminal tyrosine deletion or an indole drug. *FEBS Lett.,* **2007**, *581,* 4749-4753.

[77] Kaiser, A.; Nishi, K.; Gorin, F.A.; Walsh, D.A.; Bradbury, E.M.; Schnier, J.B. The cyclin-dependent kinase (CDK) inhibitor flavopiridol inhibits glycogen phosphorylase. *Arch. Biochem. Biophys.,* **2001**, *386,* 179-187.

[78] Oikonomakos, N.G.; Schnier, J.B.; Zographos, S.E.; Skamnaki, V.T.; Tsitsanou, K.E.; Johnson, L.N. Flavopiridol inhibits glycogen phosphorylase by binding at the inhibitor site. *J. Biol. Chem.,* **2000**, *275,* 34566-34573.

[79] Hampson, L.J.; Arden, C.; Agius, L.; Ganotidis, M.; Kosmopoulou, M.N.; Tiraidis, C.; Elemes,Y.; Sakarellos, C.; Leonidas, D.D.; Oikonomakos, N.G. Bioactivity of glycogen phosphorylase inhibitors that bind to the purine nucleoside site. *Bioorg. Med. Chem.,* **2006**, *14,* 7835-7845.

[80] Cohen, P.T. Protein phosphatase 1--targeted in many directions. *J. Cell Sci.,* **2002**, *115,* 241-256

[81] Cohen, P. The twentieth century struggle to decipher insulin signalling. *Nat. Rev. Mol. Cell Biol.*, **2006** *7,* 867-873.

[82] Aiston, S.; Coghlan, M.P.; Agius, L. Inactivation of phosphorylase is a major component of the mechanism by which insulin stimulates hepatic glycogen synthesis. *Eur. J. Biochem.,* **2003**, *270,* 2773-2781.

[83] Fosgerau, K.; Breinholt, J.; McCormack, J.G.; Westergaard, N. Evidence against glycogen cycling of gluconeogenic substrates in various liver preparations. *J. Biol. Chem.,* **2002**, *277,* 28648-28655

[84] Hampson, L.J.; Mackin, P.; Agius, L. Stimulation of glycogen synthesis and inactivation of phosphorylase in hepatocytes by serotonergic mechanisms, and counter-regulation by atypical antipsychotic drugs. *Diabetologia,* **2007**, *50,* 1743-1751.

Received: April 23, 2010 Revised: June 21, 2010 Accepted: July 12, 2010