Recent Advances in the Allosteric Inhibition of Glycogen Phosphorylase

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Abstract: Glycogen Phosphorylase (GP) is an important target for the development of anti-hyperglycaemic drugs. GP is an enzyme which is moderated allosterically with multiple ligand binding sites where inhibitors can potentially modulate enzyme activity. The search for potent and isoform selective inhibitors of GP is ongoing with an increasing focus on allosteric inhibition. In this review, the structural diversity, and enzyme interactions of the most recent inhibitors, and in particular allosteric inhibitors, of GP at the different key binding sites are explored. A range of inhibitors of GP, with known as well as unknown binding site or mechanism is presented.

Keywords: Glycogen phosphorylase, allosteric inhibition, inhibitor, structure based drug design.

Dedicated to Late Professor Nikos G. Oikonomakos, Institute of Organic and Pharmaceutical Chemistry (IOPC) of the National Hellenic Research Foundation and Head of the Structural Biology and Chemistry Group (SBCG).

1. INTRODUCTION

Glycogen Phosphorylase (GP) plays a central role in the regulation of glycogen metabolism, and has become an important therapeutic target for the prevention of high blood glucose levels [1]. GP catalyses the first step in intracellular degradation of glycogen to produce glucose-1-phosphate (glycogenolysis), and is controlled by allosteric regulation and reversible phosphorylation [2]. GP exists in three different isoforms; human liver glycogen phosphorylase (HLGP), human muscle glycogen phosphorylase (HMGP), and human brain glycogen phosphorylase (HBGP) [3], which share 80% amino acid sequence homology. Detailed alignment and comparison of amino acids residues 1-830 of the three isoforms have been reported elsewhere [4]. The ~20% nonidentical amino acid residues are distributed throughout the sequence with abrupt divergence of the three sequences around amino acid 830 [4]. The binding of glucose-1-phosphate at the catalytic site results primarily in hydrogen bonding interactions with the key amino residues Asn133, Gly134, His 376, Thr 377, His 570, Glu 671, Ser 673 and Thr 675 [5]. These residues are preserved between the three different isoforms of GP, which may account for the lack of isoform selectivity obtained with catalytic site inhibitors and suggest that alternative mechanisms of inhibition should be considered. Further analysis for the other binding sites reveals only subtle differences in the sequences between the isoforms, based on isolated amino acid residues variance. For example, amino acid residues 5-22 are involved with binding AMP and thus act as the key residues in the AMP-allosteric site [6]. Comparison of the three isoforms for residues 5-22 shows total sequence homolog for residues 6, 8-10, 12-14, 16-17 and 20, partial homology for HMGP and HLGP for residues 7, 22 and 23. Similarly the indole (new allosteric site) binding site [7] is highly conserved across isoenzymes with only a single amino-acid substitution (Ala192 in brain and muscle, Ser192 in human), whereas purine site inhibitors form synergistic binding interactions with different residues of the 280's loop which are preserved across the isoforms [8].

General inhibitors of GP also have the potential to function as antitumour drugs, preventing tumour growth by inhibiting glycogen breakdown in cancers associated with increased glycogen levels (colorectal and lung cancer) [9, 10]. Structure assisted drug design of inhibitors to control blood glucose levels represent the primary focus for studies about GP [1, 3, 11-15]. In addition, reports have indicated GP inhibition to be efficient in lowering blood glucose in diabetic models, thereby validating it as an attractive therapeutic target for type-2 diabetes [16]. A major challenge in the development of therapeutic agents for the control of blood glucose is the selective inhibition of HLGP. Low isoform specificity of GP inhibitors may lead to problems in skeletal muscle contraction and control, and central nervous system failure [17, 18]. However, recent work has suggested that glycogen phosphorylase inhibition that aimed at controlling blood glucose levels is unlikely to negatively impact on high intensity contraction of muscles, although the impact for prolonged low intensity contraction is unknown [17, 18].

2. GLYCOGEN PHOSPHORYLASE (GP)

GP is a dimeric enzyme (194 kDa for the dimeric form) of two identical subunits [2], with each subunit divided into two domains (Ser1 to Gly480 and Tyr481 to Pro841). The

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1389-5575/10 $55.00+.00

N-terminal domain Ser1 to Gly480 includes the Ser14-phosphorylation site, a glycogen storage site, the AMP/ATP site, and part of the active site. It also contains the whole intersubunit contact region through which conformational changes are coordinated between the two subunits. The C-terminal domain Tyr481 to Pro841 and residues from the N-terminal domain form the glucose and purine inhibitory site. The active site is located in a cavity at the centre of the GP molecule [2, 19-21]. The essential cofactor, pyridoxal-5'-phosphate (PLP), is linked by a Schiff base to Lys680 located into the catalytic site [11].

GP exists in two forms which interconvert, GPa and GPb. GPb is converted to GPa by phosphorylation catalysed by the enzyme phosphorylase kinase. In turn dephosphorylation catalysed by protein phosphatase 1 (PP1, an enzyme regulated in response to insulin) converts GPa back to GPb. GPa and GPb each exist in equilibrium between a less active T (T for "tense") and a more active R-state (R for "relaxed") (Fig. **1**) [11]. GPa exists predominantly in the high activity R-state, whereas GPb primarily exists in the less active T-state. GP is highly regulated by allosteric control. AMP is an allosteric activator (binds to a site other than the catalytic site) of GP and converts the inactive T-states of GPa and GPb to the active R-states. In turn, allosteric inhibitors such as ATP, glucose-6-phosphate, glucose and caffeine can alter the equilibrium back to the inactive T-states of GP [19]. Studies have shown that conversion of the GPb T-state to GPa R-state by phosphorylation brings about a conformational change in the enzyme, due to the changes in the tertiary structure [20]. In the T-state, access to the catalytic site is partly blocked by the 280's loop. On transition from the T to R-state, the 280's loop is disordered and a channel opens that allows a crucial arginine to enter the catalytic site in place of an aspartic acid unit, creating the phosphate recognition site [2, 11]. In addition, the open channel also allows the glycogen substrate to reach the catalytic site [11]. The same conformational change occurs when an allosteric activator such as AMP binds to the T-state of GPa or GPb, converting them to their active R-states. A more detailed description of the structure and related functions of GPa has been published elsewhere [22].

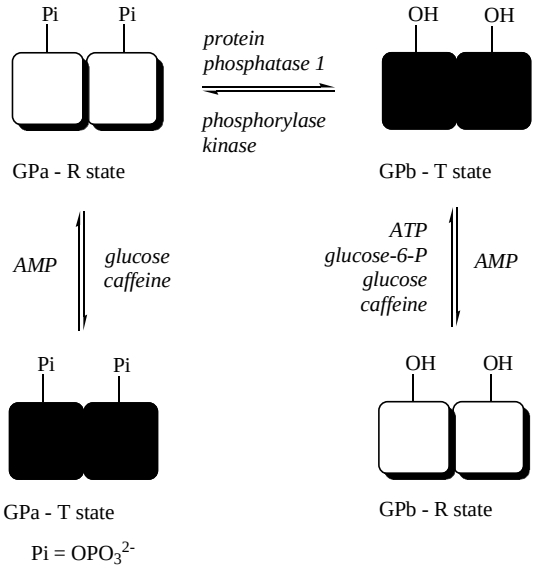


Fig. (1). Representation of the allosteric transitions of glycogen phosphorylase. Adapted from Ref [11].

3. THE BINDING SITES AND INHIBITION OF GP

Several binding sites for GP have been identified. The *catalytic* site binds the substrates glucose-1-phosphate and glycogen, and the inhibitors glucose and glucose analogues. The *AMP-allosteric site* binds the activator AMP and the inhibitors ATP and glucose-6-P-phosphate. The new *allosteric indole site* occurs at the dimer interface between the two subunits. The *inhibitor site*, which is also referred to as the purine site or nucleoside site, binds caffeine and related compounds. In addition there are the *phosphate binding site* and the *glycogen storage site*, which bind glycogen (Fig. **2**) [6, 11, 13, 23]. The first four of these regulatory sites are known to be inhibitor binding sites.

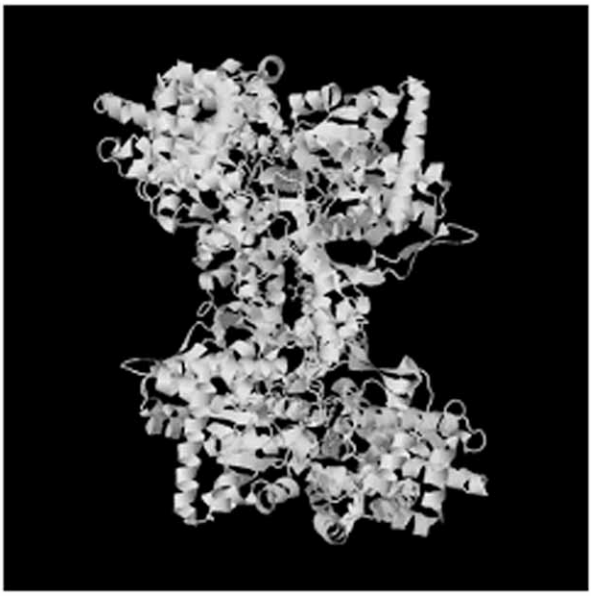


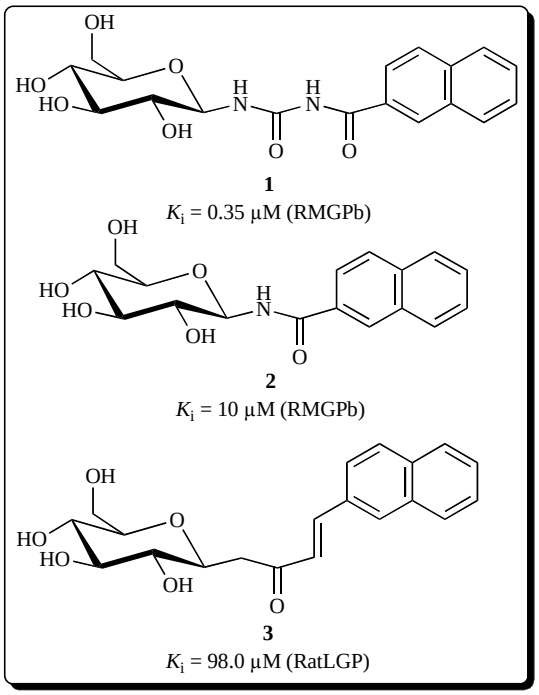
Fig. (2). Schematic diagram of the GP dimeric molecule viewed down the molecular diad. General locations of the catalytic and allosteric regulatory sites of glycogen phosphorylase are shown (Adapted from PDB entry 1K06).

Typically catalytic site inhibitors form the focus of drug-development aimed at key enzymes. By targeting such a highly conserved site within an enzyme, the variation in molecular size, stereochemistry and functionality of an inhibitor compound is usually very restricted. For GP, a range of structurally diverse molecules has been identified, due to the high degree of allosteric regulation of GP. In this review different species (rabbit, rat, pig or human), tissue isoforms (liver, muscle or brain,), and states of GP (GPa, GPb) have been used in GP inhibition assays [10, 24]. Some compounds show analogous inhibitory activity independent of the species, phosphorylation state, or tissue isoform used, however dramatic differences in inhibition of GP have also been reported [25]. The presence and absence of an allosteric ligand such as glucose and AMP can influence the measure of inhibitory activity [24, 25]. Thus, caution should be taken

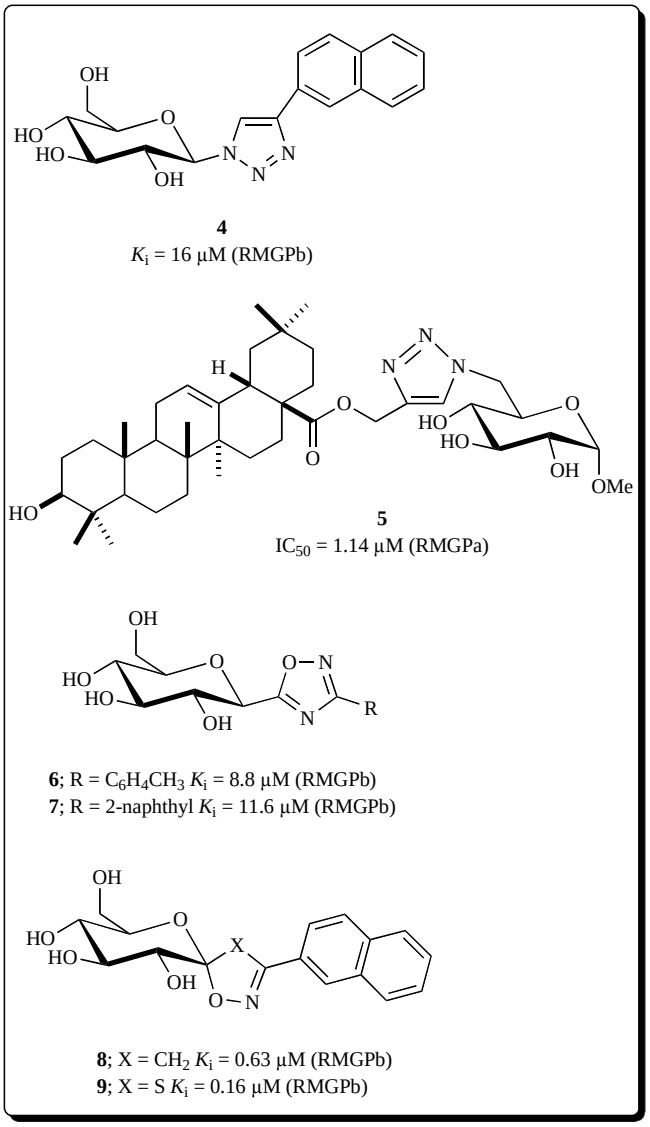
when comparing levels of inhibition measured under non-identical conditions [10]. The species, isoform and state of GP used to measure the inhibitory activity for the discussed compounds will be quoted where it is available. Extensive research has been carried out in recent years to find new potent inhibitors for GP. Comprehensive reviews on a range of GP inhibitors and their proposed sites of action have been reported elsewhere, to which the reader is referred [3, 11, 14, 24-28]. Recently, work on allosteric inhibitors is coming to the fore. In this review, a summary of the literature up to and including February 2010 in this area will be presented, with a focus on allosteric inhibitors.

4. CATALYTIC SITE INHIBITORS

The catalytic site is located in a cavity at the centre of the GP molecule, 15 Å from the surface [29, 30]. Many catalytic site inhibitors are based on the physiological inhibitor glucose and this area has been extensively reviewed [11, 14, 24-38]. Innovations in the last few years include 1-(β-D-glucopyranosyl)acylated ureas, exemplified by compound (**1**) [39], N-(β-D-glucopyranosyl)amides with aromatic groups, as shown in compound (**2**) [40] and polyfunctional C-alkanoyl glycosides, such as compound (**3**) [41, 42]. Notably from these studies, it was identified that the π-donor ability of the 2-naphthyl group of (**2**) was essential for the strong inhibition by N-(β-D glucopyranosyl)amides [40].



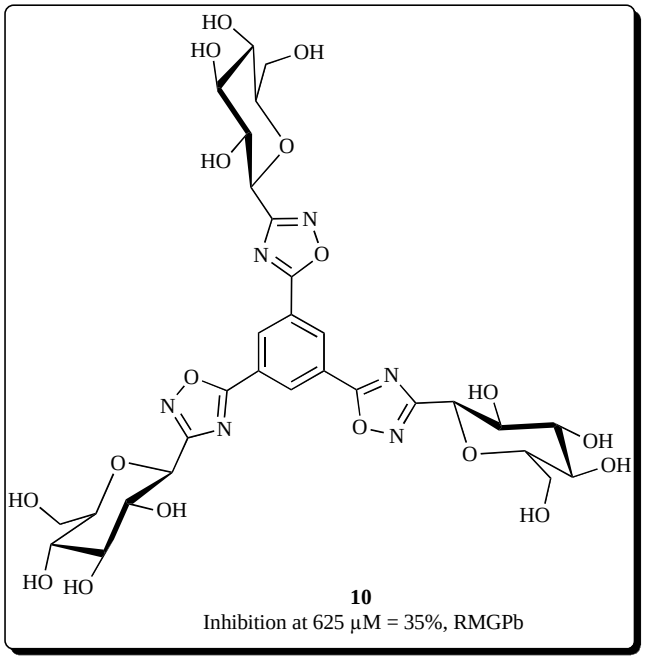
The use of a 1,2,3-triazole moiety as a bioisosteric replacement [43-47] for the amide has also emerged. The binding of inhibitors, such as compound (**4**) (K_i = 16 μM, RMGPb), occurs at the catalytic site while the substituent group in the β-position of the C1 atom makes additional hydrogen bonding and van der Waals interactions to the protein [46, 48]. Similar studies have established equipotent or more potent compounds such as glucoconjugates of oleanolic acid, linked by a triazole moiety, such as (**5**) (IC_{50} value of 1.14 μM, RMGPa) [49], 5-β-D-glucopyranosyl-3-(4-methyl-phenyl- and -2-naphthyl)-1,2,4-oxadiazoles (**6**) and (**7**) (K_i = 8.8 and 11.6 μM, respectively, RMGPb) [50], naphthyl glucopyranosylidene-spiro-isoxazoline (**8**) (K_i = 0.63 RMGPb) [51], and 2-naphthyl-substituted glucopyranosylidene-spiro-1,4,2-oxathiazole (**9**) (RMGPb (K_i = 160 nM) [52]. Compound (**9**) is one of the most potent glucose-based inhibitors currently known.



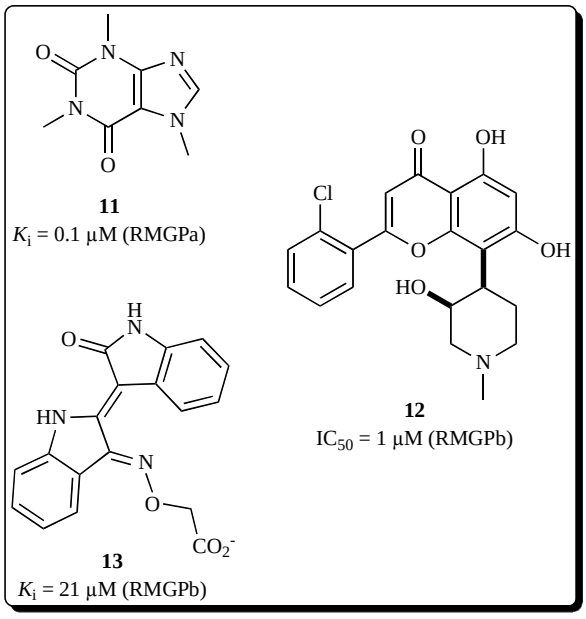
Monovalent and trivalent C-glycosylated oxadiazoles derivatives, such as (**10**), are one of the few examples of multivalent inhibition for an enzyme. However, compound (**10**) only displays moderate inhibition (35% at 625 μM, RMGPb) of GP, where the multivalent inhibitors were more potent than their monovalent counterparts [53]. In combination, these results indicate the importance of extensive interactions of the inhibitors in the β-channel of GP, which occur largely through the aglycon moiety.

5. PURINE NUCLEOSIDE SITE INHIBITORS

The purine nucleoside binding site is situated about 12 Å from the catalytic site. Located at the entrance of the cavity that forms the catalytic site, the purine nucleoside binding



10
Inhibition at 625 μM = 35%, RMGPb



11
K_i = 0.1 μM (RMGPa)

12
IC_{50} = 1 μM (RMGPb)

13
K_i = 21 μM (RMGPb)

site is made up of amino acid residues from both C- and N-domains (Phe280 from the 280's loop and Tyr613 from the α19 helix), forming a hydrophobic pocket [11, 14]. Inhibitors that bind at this site tend to undergo π-π stacking between aromatic residues of the inhibitor and Phe285 and Tyr613, which stabilise the inactive T-state of GP, and block access to the catalytic site. The major source of binding energy for a known ligand comes from the π-π stacking, which suggests that a wide range of aromatic compounds could bind to this site [54].

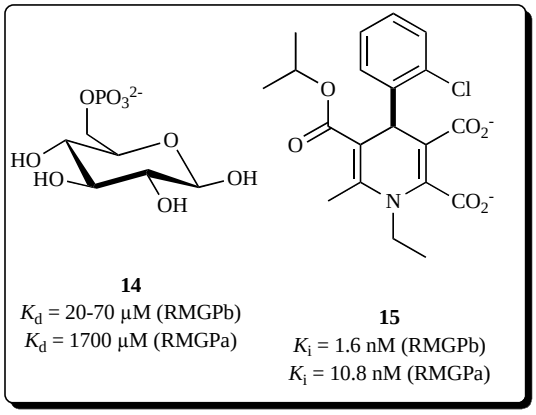
Known ligands that bind at this site include; purines (e.g. caffeine (K_i = 0.1mM, RMGPa) (**11**) [55-57] and adenine), flavopiridol (**12**) (IC_{50} = 1μM and K_i = 1μM; one of the most potent inhibitors at this site), nucleosides (e.g. adenosine), and nucleotides (e.g. AMP, IMP, ATP, NADH and FAD). All of these inhibitors display synergism with glucose [11, 24], which in part explains why these relatively weak inhibitors are still quite effective. One structure activity relationship (SAR) study, reported eighteen inhibitors of this site, most of which were physiological compounds [13]. Some other known inhibitors are riboflavin [13], piperidinyl-4H-benzopyran-4-ones [58], and indirubin derivatives, such as (**13**) [59, 60]. Compound (**13**) has been shown to be a competitive inhibitor of GPb with respect to both Glc-1-P and AMP. The crystal structure of the GPb-(**13**) complex showed one ligand molecule of (**13**) bound at the inhibitor purine site. Compound (**13**) was intercalated between the two aromatic side chains of Phe285 and Tyr613 [59] with the indole-aminooxy-acetate rings of (**13**) inclined at approximately 40˚ [59]. In addition, two molecules of (**13**) were also bound at the allosteric activator AMP binding site and a subsite in the vicinity of the allosteric site. The inhibitor site has remained relatively unexplored with little work reported in the literature on the development and optimisation of inhibitors for this site. A recent publication suggested that analogues of (**12**) fail to induce glycogen synthesis, which perhaps explains the recent lack of interest for this site [61].

6. ALLOSTERIC SITE (OR AMP BINDING SITE) INHIBITORS

The allosteric site (or AMP binding site) is located about 35 Å from the catalytic site, where the helices α2 (residues 47-78) and α8 (residues 289-314) form a V shape [11, 62]. The site is lined by β-strands from the central core of β4 (residues 153-160), β11 (residues 237-247) and surrounded on a third side by β7 (residues 191-193) plus the following loop to residue 197. The three-sided channel is closed at one end by the cap' region (residues 36'-47'; the superscript prime refers to residues from the opposing subunit of the GP dimer) [63]. The allosteric site binds AMP and a range of other phosphorylated compounds, such as glucose-6-P (**14**) which is the most potent physiological inhibitor of GPb (K_d = 20-70 μM). However, the inhibitor capacity of (**14**) is much less effective when competing with AMP or when binding to the R-state GPa [64, 65]. The binding of (**14**) to the allosteric site occurs mostly through hydrogen bonding and a few van der Waals interactions. Upon binding of (**14**) a conformational change occurs that results in a modified, but still inactive, T-state [63, 66].

The AMP allosteric site has shown a remarkable ability to accept a diverse number of chemotypes for binding at this site. There appears to be protein flexibility in the site allowing it to use the same amino acid residues for binding dissimilar compounds, mainly through ionic interactions [11]. BAY W1807 (**15**) (K_i = 1.6 nM for GPb and K_i = 10.8 nM for GPa) was, until a few years ago, the most potent inhibitor of GP. BAY W1807 (**15**) is an *in situ* product of oxidation once the pro-drug lactone reaches the liver. BAY W1807 (**15**) stays in the liver as the diacid form. Tissue selectivity for the liver is thus achieved through this oxidative mechanism. BAY W1807 (**15**) acts in synergy with glucose. This is an important feature for GP inhibitors as the potency of the inhibitor would decrease along with the glucose concentration resulting in a diminishing risk for the common side effect, hypoglycaemia [11, 67]. BAY W1807 (**15**) exploits numerous contacts with the AMP allosteric site. The 5,6-

dicarboxylic acid moiety of (**15**) mimics the phosphate group found in glucose-6-P (**14**) and forms hydrogen bonds to the three arginine residues Arg309, Arg310 and Arg242 in the phosphate recognition site [24, 25]. The chlorine atom of (**15**) fits in a pocket where it makes van der Waals interactions with the amino acid residues Arg193 and Asp227. The ethyl and isopropyl groups make contact with the amino acid residues Tyr75, Trp67, Ile68 and Arg193 and a π-π-stacking interaction is seen between the aromatic rings and the aromatic amino acid residues in the allosteric site [11, 68-69].

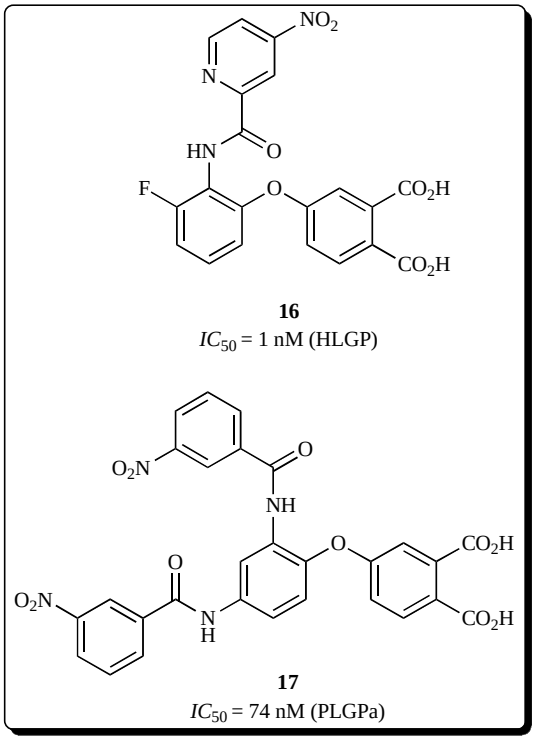


14
K_d = 20-70 μM (RMGPb)
K_d = 1700 μM (RMGPa)

15
K_i = 1.6 nM (RMGPb)
K_i = 10.8 nM (RMGPa)

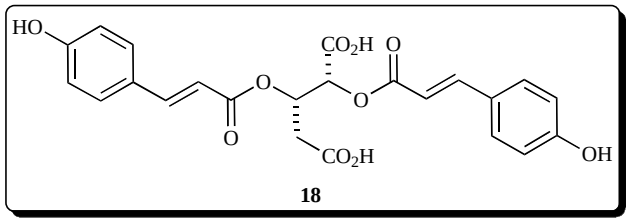
Within the last 10 years a new class of diacid analogue with improved potency has been reported [15, 67], showing improved potency to previously reported GP inhibitors. The best compound was phenyl diacid (**16**) (IC_{50} = 1 nM), along with another diacid which was identified through high-throughput screening (HTS), compound (**17**) (IC_{50} = 74 nM pig liver glycogen phosphorylase; PLGP) [67]. Phenyl diacid (**16**) showed a similar binding mode to that of BAY W1807 (**15**), as established by both molecular modelling and X-ray crystallography data [62]. The structural studies indicated that the pyridyl nitrogen was important for binding and suggested that the nitrogen atom facilitated beneficial interactions made to GP by changing the electron distribution. In addition, conformers of the phenyl diacid series bind to the allosteric site with a "V-shape" stabilised by a hydrogen bond between the amide NH and the pyridine nitrogen [62]. This "V-shape" allows interaction to the same Arg309 and Arg310 as Bayer diacid W1807 (**15**), however the orientation of (**16**) is perpendicular to that of (**15**), giving an interaction to Arg81 instead of Arg242, as for (**15**). This highlights the remarkable flexibility of this binding site [15, 62].

Further exploration of potential inhibitors for the AMP allosteric site includes other dicarboxylic acids analogues [15, 16, 67], acyl ureas [70-72], [(arylamido)phenoxy] phthalic acids [62], and indirubin-3'-aminooxy-acetate (**13**) [59]. The allosteric site tolerates diversity in the structure of the inhibitors for this site. This is illustrated by FR258900 (**18**), which has little structural similarity to other inhibitors of the AMP site. FR258900 (**18**) was isolated from the fungal strain No. 13835 and inhibits HLGPa [73], and lowers plasma glucose levels in animal models of diabetes [74]. FR258900 (**18**), binds to the allosteric site by non-polar van der Waals interactions to Gln71, Gln72, Phe196, and Val45', and also by ionic interactions from the carboxylate groups to the three arginine residues Arg242, Arg309 and Arg309 [75].



16
IC_{50} = 1 nM (HLGP)

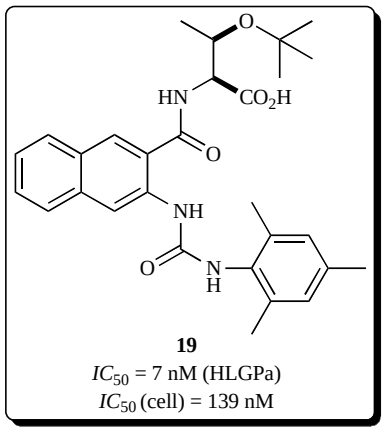
17
IC_{50} = 74 nM (PLGPa)



18

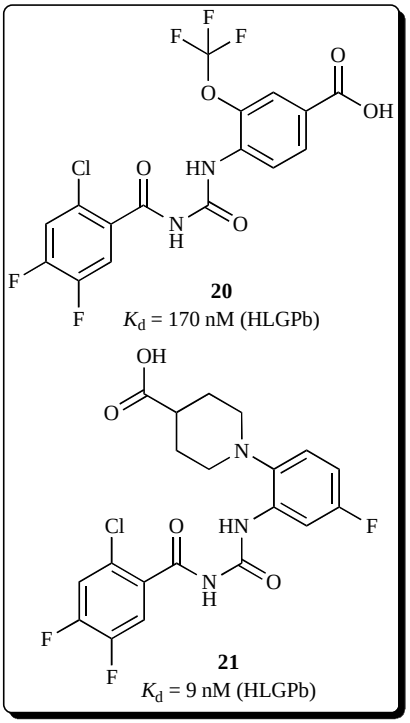
Recent work on allosteric site inhibitors includes the optimisation of the amino acid residue of a series of serine and threonine ether amino acid anthranilimide-based GP inhibitors [76-79]. Modification of the amino-acid group through a systematic SAR showed how overall low nanomolar potency for GPa inhibition could be obtained. *t*-Butylthreonine 2,4,6-trimethylphenyl anthranilimide analogue (**19**) displayed potent *in vitro* inhibition of GPa (IC_{50} = 7nM; IC_{50} (cell) = 139 nM), as well as *in vivo* efficacy in a diabetic model [77]. The X-ray crystal structure of compound (**19**) [78] bound to GPa revealed a number of important hydrogen bonds. A bidentate interaction occurs between the carboxylate of the amino-acid with Arg310 of the phosphate- recognition site. A hydrogen bond is formed between the amide carbonyl and the NH_2 of Gln71. Two hydrogen bonds are formed between the urea of (**19**) and the main-chain of the symmetry-related subunit of the homo-dimer; (1) the urea NH with the carbonyl of Val40' and (2) the urea carbonyl with the NH of Asp42'. The lipophilic regions of the amino-acid are surrounded by Phe196, the alkyl chain of Arg309 and Ala313, whereas the naphthalene moiety of (**19**) is surrounded by residues Asp42', Asn44' and Val45' as well as Ile68, Gln71, Gln72 and Tyr75. The urea phenyl ring is situated deep in the binding site. Notably, the conformation of GP when bound to the inhibitor of (**19**) is different to that observed for the other reported AMP site binders [66]. The glucose lowering ability

of compound (**19**) in a diabetic mouse model (ob/ob) was measured at 5 mg/kg. Compound (**19**) gave a 25 % reduction in the 2 hr glucose response curve [78].



19
IC_{50} = 7 nM (HLGPa)
IC_{50} (cell) = 139 nM

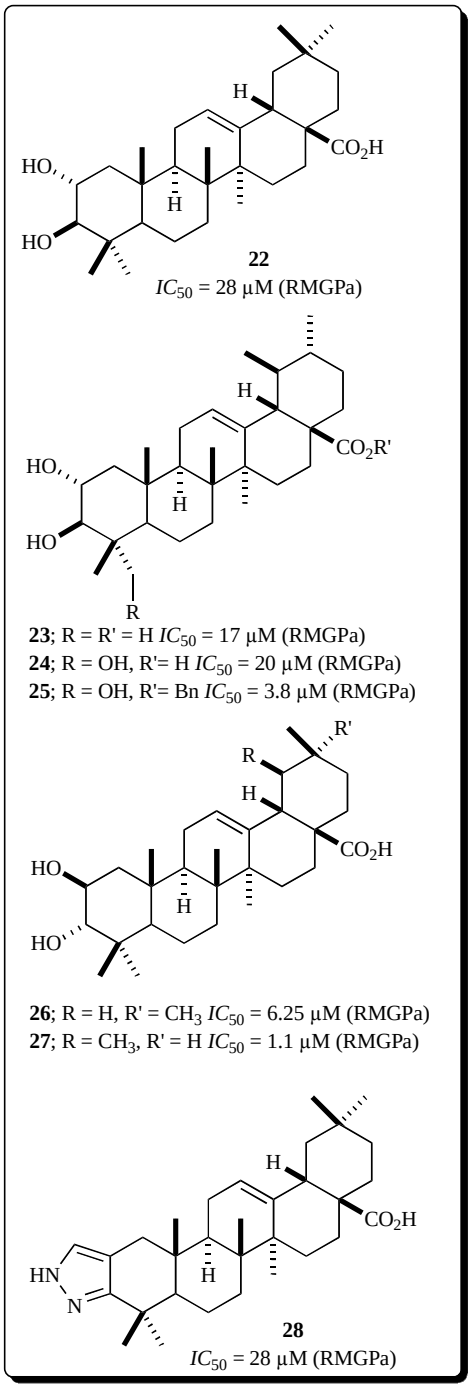
In a related acyl urea series, AVE5688 (**20**) and AVE2865 (**21**) were identified as highly potent GP inhibitors that compete with the physiological activator AMP and act synergistically with glucose. Crystal structures confirmed that (**20**) and (**21**) bind to the lower part and full AMP site of T-state GP respectively. This provided direct evidence that acyl urea and quinolone compounds are allosteric inhibitors that selectively bind to and stabilize the inactive conformation of the enzyme. Different thermodynamic contributions to inhibitor potency were identified that were related to the binding modes observed in the individual co-crystal structures of (**20**) and (**21**) with HLGPa [80].



20
K_d = 170 nM (HLGPb)

21
K_d = 9 nM (HLGPb)

Another class of inhibitors of the allosteric site are the pentacyclic triterpenoids maslinic acid (**22**), asiatic acid (**23**), corosolic acid (**24**), and related compounds. The crystal structures of the RMGPb-asiatic acid (**23**) [81] and RMGPb-maslinic acid (**22**) complexes [28] demonstrated that (**22**) and (**23**) bind at the allosteric site. This stimulated further work on pentacyclic terpenes, which has been described in recent reviews [27, 28]. Examples include the SAR studies of synthetically derived derivatives of corosolic acid, and maslinic acid [82-85]. Key findings from these studies included identification of the importance of inclusion of a 2,3-hydroxyl group for binding, and that esterification of the 3-β-OH group with a large hydrophobic leads to improved inhibition.



22
IC_{50} = 28 μM (RMGPa)

23; R = R' = H IC_{50} = 17 μM (RMGPa)
24; R = OH, R'= H IC_{50} = 20 μM (RMGPa)
25; R = OH, R'= Bn IC_{50} = 3.8 μM (RMGPa)

26; R = H, R' = CH_3 IC_{50} = 6.25 μM (RMGPa)
27; R = CH_3, R' = H IC_{50} = 1.1 μM (RMGPa)

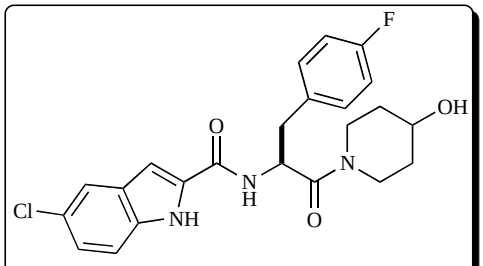
28
IC_{50} = 28 μM (RMGPa)

In other studies the SAR of asiatic acid derivatives has been explored in a series of synthetic derivatives. Asiatic acid benzyl ester (**25**) (IC_{50} = 3.8 μM) was more potent than

asiatic acid (**23**) (IC_{50} = 17 μM). SAR analysis showed that the spatial orientation of the 2-OH group was important. Comparison of asiatic acid (**23**) which possesses a 2α-OH group and eriantic acid B which possesses a 2β-OH showed asiatic acid (**23**) to be more potent against GP [86]. Other pentacyclic triterpene inhibitors that have been identified include; isomeric 2,3-dihydroxy lupanes of alphitolic acid which exhibited moderate inhibitory activity against RMGPa [87]; a series of 23-hydroxybetulinic acid derivatives [88]; bredemolic acid (**26**) (IC_{50} = 6.25 μM, RMGPa) [89], the naturally occurring 2β, 3α-isomer of maslinic acid (**22**); 2β, 3α-dihydroxyurs-12-en-28-oic acid (**27**), which is the 2β, 3α-isomer of corosolic acid (**24**) (IC_{50} = 1.1 μM; RMGPa) [89]; pyrazolo[4,3-b]oleanane derivatives, such as (**28**) [90]; novel pentacyclic triterpenes [91] and 3-deoxy-2-keto derivatives of oleanolic acid and ursolic acid, such as 2R-hydroxyurs-12-en-28-oic acid (IC_{50} = 1.2 μM; RMGPa) [92]. It is interesting to note that to date none of the pentacyclic terpenes have achieved nM inhibition values against GP. As can be seen from the summary above, the structural diversity in the inhibitors of the allosteric site is significant. This reveals the remarkable flexibility of this binding site and the importance of a range of lead compounds for inhibition at this site.

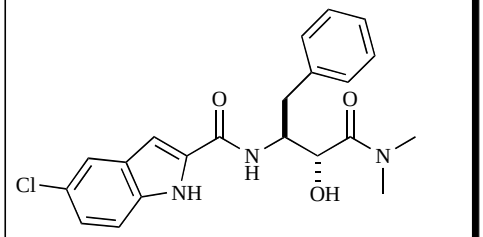
7. INDOLE SITE (NEW ALLOSTERIC SITE) INHIBITORS

The indole binding site is located about 33 Å from the catalytic site and 15 Å from the AMP allosteric site. The site is situated in a 30 Å long cavity at the dimer interface of the two GP subunits [24]. There is one indole site on each subunit and in the native T-state of GPb the cavity they form is occupied by 60 water molecules (30 molecules in each subunit). Only 8 out of 60 water molecules make contact with the enzyme through hydrogen bonds. This site is referred to as the indole site since almost all inhibitors that bind to the site have been based on an indole-2-carboxamide scaffold, as exemplified by CP-320,626 (**29**).



29

IC_{50} = 205 nM (HLGP)



30

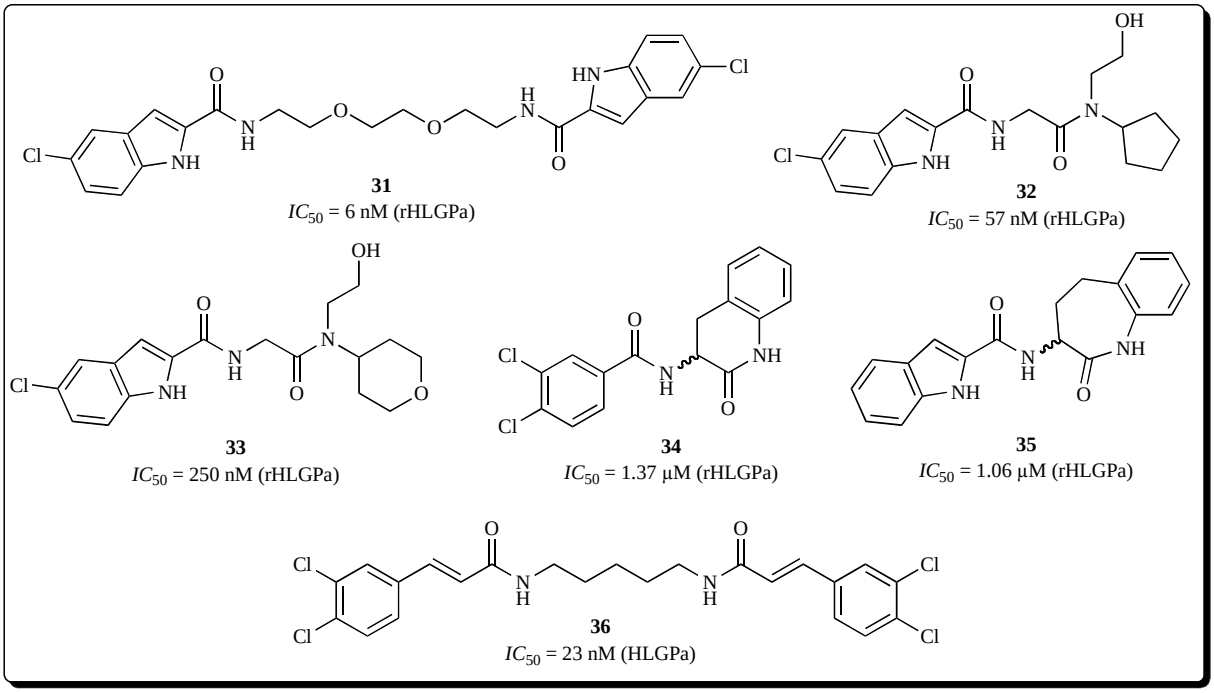
IC_{50} = 110 nM (rHLGP)

The indole binding site is made up of amino acid residues from both subunits giving two different environments, one that is hydrophobic where the indole moiety binds, and one that is both hydrophobic and polar, and extends into the solvent filled cavity (where the carboxamide binds) [6]. CP-320,626 (**29**) (IC_{50} = 205 nM for HLGP and IC_{50} = 83 nM for HMGP) is an inhibitor that binds the indole site and thereby inhibits GP by stabilising a slightly modified, but still inactive, T-state [23]. Indole (**29**) showed oral activity (marked blood glucose lowering effect at 10 mg/kg compared to 25-50 mg/kg for (**30**), in diabetic *ob/ob* mice), synergistic binding with caffeine and glucose, and no effect on insulin secretion in isolated rat islets [93]. In addition, (**29**) inhibited recombinant human muscle glycogen phosphorylase (rHMGPa) [93] (IC_{50} = 83 nM) but did not substantially impair glycogen mobilisation, *in situ*, under the conditions where glycogenolysis would be inhibited. This may be due to low inhibitor concentrations in the muscle cells [93].

GPb interacts with CP-320,626 (**29**) through seven hydrogen bonds and 114 van der Waals bonds [35]. The stronger interactions are mainly aromatic/aromatic and amino/aromatic. The 4-fluoro-benzyl group interacts with the side chain of a phenylalanine residue (Phe53) and the 4-fluoro-benzyl group interacts with the histidine residue His67. Also, hydrogen bonds are formed between the indole nitrogen and a carbonyl on the backbone of the enzyme, and between the 2-carboxamide carbonyl and the threonine amino acid residue Thr38 in the indole site [6, 11, 23]. X-ray crystallography studies have shown that two molecules of the indole inhibitor bind identically, within 6 Å of each other, in the two subunits. This discovery leads to the synthesis of CP-526,423 (**31**) (IC_{50} = 6 nM), a potent dimer that contains two 5-chloroindole-2-carboxamide moieties joined by an ethylene glycol linker [6]. X-ray crystallography data has shown that CP-526,423 (**31**) spans the two indole binding sites [6]. It has been suggested that the solvent filled gap including the indole site may be necessary to allow for the rotation between the two subunits that activates GP, and that the bis-indole (**31**) binding to both the dimers is more successful at stopping this rotation. Bis-indole (**31**) is one of the most potent known allosteric indole inhibitors [6].

Further modifications of the indole-2-carboxamide scaffold have led to the discovery of numerous indole compounds that show inhibitory activity [6, 12, 23, 93-108]. Two examples of this series are chloroindole (**32**) (IC_{50} = 57 nM, rHLGPa) and (**33**) (IC_{50} = 250 nM, rHLGPa), which gave similar or improved potency as well as a marked blood glucose lowering effect at 5 mg/kg in diabetic *ob/ob* mice [108], as compared to (**30**) (IC_{50} = 110 nM, rHLGPa and 25-50 mg/kg) [93]. A crystal structure (PDB accession code: 1XOI) of (**32**) with rHLGPa showed that (**32**) binds to the same allosteric inhibitor site [108], utilising similar interactions as described for previous chloroindole inhibitors [6]. Compounds from the chloroindole series reached phase II clinical trials [1, 24, 93, 109]. However, the development has been discontinued, may be due to poor selectivity for liver GP over muscle GP, as *in vivo* tests have shown glycogen accumulation in both liver and muscle [24].

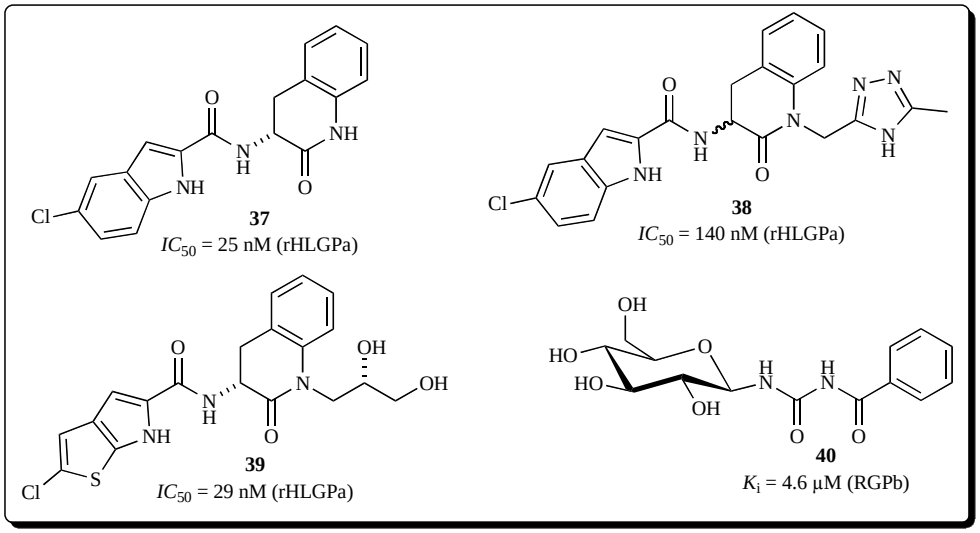
Extensive work within the chloroindole series has exhausted the data that can be extracted from this scaffold and



has prompted the search for other compounds that mimic the indole scaffold or identify a structurally different scaffold. New lead compounds have been identified through HTS and include (**34**) (IC_{50} = 1.37 μM, rHLGPa), where the 3,4 dichlorobenzamide acts as an indole analogue and (**35**) (IC_{50} = 1.06 μM, rHLGPa). Compounds (**34**) and (**35**) have comparable potency for both rHLGPa and rHMGPa [12], whereas bis-3-(3,4-dichlorophenyl) acrylamide (**36**) (IC_{50} = 0.023 μM) inhibited HLGPa with improved potency. An X-ray crystallographic study of the enzyme–(**36**) complex showed that the 3,4-dichlorophenyl moiety interacts through hydrophobic interactions with the enzyme with the inhibitor bound at the dimer interface site [110].

Combination of the 6-chloroindole moiety with the six-membered lactam ring gave rise to potent analogues, (**37**) (IC_{50} = 25 nM, rHLGPa, EC_{50} = 1.8 μM, rat hepatocytes) and (**38**) (IC_{50} = 140 nM, rHLGPa, EC_{50} = 1.2 μM, rat hepatocytes). The more polar triazolylmethyl lactam (**38**) lost some enzyme based potency while gaining cell-based potency due to improved cell penetration [12]. 2-Chloro-6H-thieno[2,3-b]pyrroles, as new indole analogues and as part of a series of substituted 3,4-dihydro-2-quinolone GP inhibitors, have also been synthesised. Good enzyme inhibitory potency but poor physical properties were initially observed [111]. Optimisation of the 1-substituent led to 2,3-dihydroxypropyl compounds, such as (**39**), which showed good *in vitro* potency and improved physical properties, together with good DMPK profiles and acute *in vivo* efficacy in a rat model [111].

Thieno[2,3-b]pyrroles have been used as a scaffold which mimics the indole moiety. X-ray crystallographic data of selected thieno[2,3-b]pyrroles showed an unexpected va-



riety of binding orientations at the dimer interface site. The binding of the thieno[2,3-*b*]pyrrole group showed similarities to structures previously reported for 5-chloroindole derivatives, with two molecules of inhibitor per molecule of GPb dimer, and the thienopyrrole moiety binding into a hydrophobic pocket defined by the residues Val40 (from the one subunit) and Val64', Arg60', Leu63', Trp67', Lys191' and Pro229' (from the other subunit) [111].

There are a few examples of other non-indole compounds that bind to the indole allosteric site. These are represented by: glucose analogues N-acyl-N'-β-D-glucopyranosyl ureas [14], such as N-(benzoyl)-N'-(β-D-glucopyranosyl)urea (**40**) (IC_{50} = 0.4 μM) [34, 112-114], 2-(β-D-glucopyranosyl) benzimidazole [32], and bicyclic sugars [115]. A more detailed discussion of non-indole compounds that bind to the indole allosteric site, reported prior to 2008, is described elsewhere [27]. In addition, numerous patents exist on the synthesis and use of allosteric indole inhibitors for GP and the reader is referred to the patent literature [26, 95-106, 116-117].

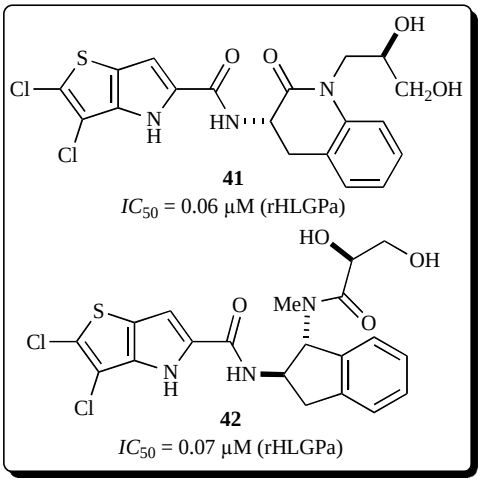
8. GP INHIBITORS WITH UNKNOWN BINDING MODE

In addition to the above mentioned inhibitor classes, there are examples of compounds that inhibit GP for which the binding site or mechanism has not been elucidated. These compounds show structural diversity ranging from similarity to existing glucose, indole and purine inhibitors to completely new structural classes. For clarity in discussing the growing numbers of other GP inhibitors, they will be presented as indole site inhibitors analogues (based on strong structural similarities with existing inhibitors of this site) and unclassified compounds. Work with such inhibitors has primarily been done through synthetic SAR studies. However, recently matched molecular pair analysis [118] (MMPA) has also been used to compare IC_{50}, solubility, and plasma protein binding for three series of GP inhibitors [111, 119-120] and to determine the limits for potency gains that can be achieved with specific structural changes.

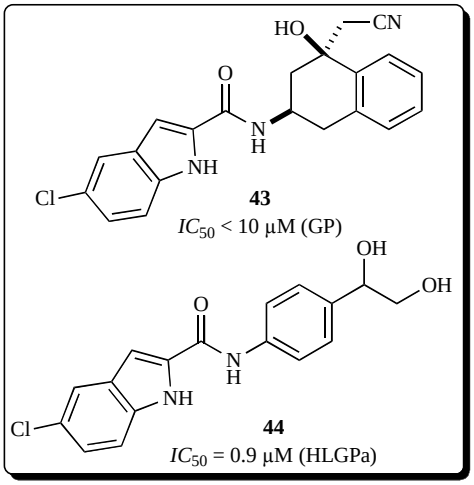
8.1. Indole Site Inhibitors Analogues

Thieno[3,2-b]pyrrole amide derivatives as indolyl surrogates [102, 111, 119, 121-127] have been prepared as GP inhibitors. For example, 1-substituted-3,4-dihydro-2-quinolone (**41**) [111] was derived in a multi-step synthesis starting from 4,5-dichlorothiophene-2-carboxaldehyde and thieno [3,2-b]pyrrole-5-carboxamide (**42**) [127] from the reaction of methyl 2-chlorothiophene-3-carboxaldehyde with methyl azidoacetate. Compounds (**41**) and (**42**) showed an enzyme inhibition constant IC_{50} of 0.06 μM and 0.07 μM, respectively [111, 127].

In the patent literature the chloro indol-2-yl moiety has been combined with benzo[b][1,4]dioxepin-7-yl moieties [117], tetrahydro-1,1-dioxide-2H-thiopyrans [123], lactams and triglyceride like prodrugs [128, 129], 5-methoxy-3,4-dihydrocarbostyril [128, 129] and tetrahydro-1H-1-benzazepin-2-ones [128] to give compounds which inhibit GP (no activity data given). Likewise, heteroaroylaminotetralins derived from 5-chloroindole-2-carboxylic acid [105, 130, 131], such as (**43**) ($IC_{50} \leq 10\,\mu M$), and other various N-bicyclo-5-chloro-1H-indole-2-carboxamide derivatives have



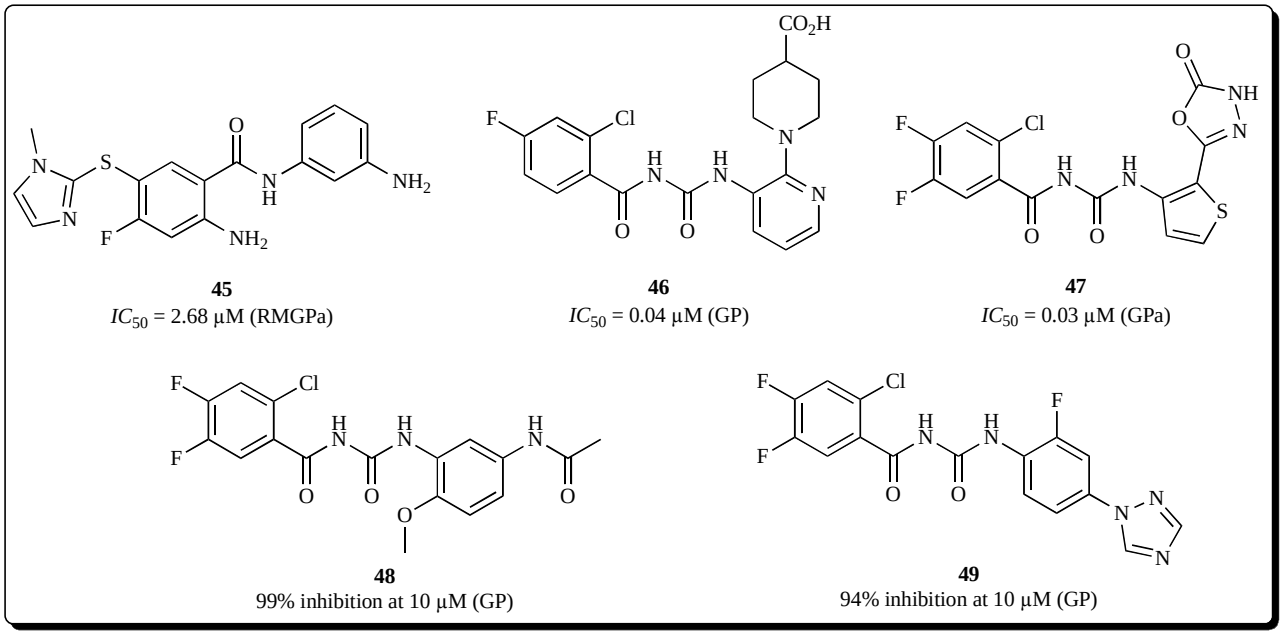
been reported. 5-Chloro-N-[4-(1,2-dihydroxyethyl)phenyl]-1H-indole-2-carboxamide (**44**), inhibited HLGPa (IC_{50} = 0.90 μM), and displayed oral hypoglycaemic activity in diabetic db/db mice. Crystallographic determination of the complex of (**44**) with HLGPa showed that the two hydroxyl groups make favourable electrostatic interactions with HLGPa and that the inhibitor binds in a solvent cavity at the dimer interface [132].



8.2. Unclassified Compounds

There is a diverse range of unclassified compounds that displays a level of inhibitory activity against GP, but the GP binding site or mechanism of inhibition has not been established. Potency of such inhibitors is often comparable to existing GP allosteric inhibitors, which may in part explain why the GP binding site has not been established for such inhibitors. The unclassified inhibitors will be presented and grouped by the structural features of the compound, such as aromatic, heterocyclic or peptide moieties.

A series of novel benzamide derivatives have been evaluated through SAR study [133]. The substituted benzamide (**45**) (IC_{50} = 2.68 μM) was identified as nearly 100 times more potent than the initial lead compound. Analysis of mapping between pharmacophores of different binding sites and each compound demonstrated that benzamide de-



45
IC_{50} = 2.68 μM (RMGPa)

46
IC_{50} = 0.04 μM (GP)

47
IC_{50} = 0.03 μM (GPa)

48
99% inhibition at 10 μM (GP)

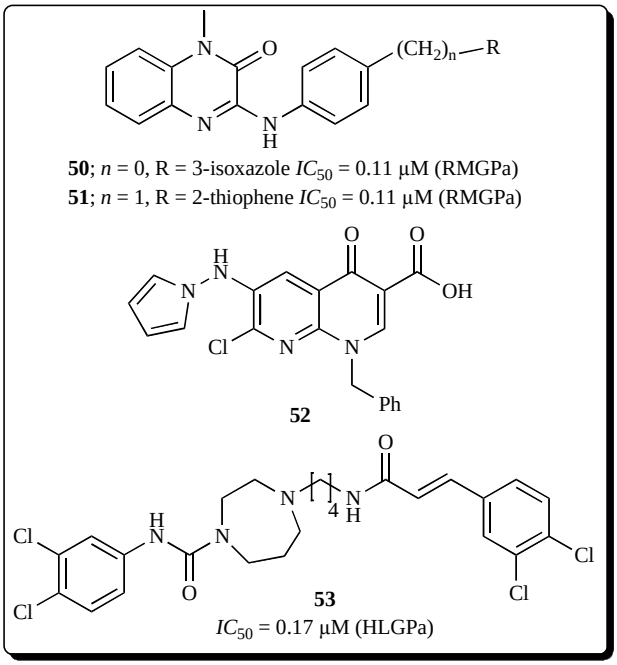
49
94% inhibition at 10 μM (GP)

rivatives from the series bind at the dimer interface of the RMGP. Possible docking modes of compound (**45**) were explored by molecular docking simulation [133].

An extension of the amide linker led to a range of benzoyl ureas which feature in the patent literature and include; benzoylureidopyridylpiperidines such as ureidopyridylpiperidine (**46**) (IC_{50} = 0.04 μM) [134]; 3-(benzoylureido)thiophenes such as 5-oxo-4,5-dihydro-[1,3,4]-oxadiazol-2-yl)thiophen-3-yl]urea (**47**) (IC_{50} = 0.03 μM) [135]; carbonylamino substituted acyl phenyl ureas, such as (**48**) (99% inhibition against GP at 10 μM) [136], and heterocyclylbenzoylureas, such as 2-fluoro-4-[1,2,4]triazol-1-ylphenyl)urea (**49**). Compound (**49**) gave 94% inhibition of activated GP at 10 μM [137].

Diversification of the heterocyclic scaffold component has included compounds such as 3-anilino-quinoxalinones [138, 139]. Compounds (**50**) and (**51**) have been shown to be effective against GP *in vitro*. SAR studies demonstrated that the structural features of the aniline moiety were most important for potent inhibitory activity, while the quinoxalinone ring was more tolerant to substitution. Compounds (**50**) and (**51**) were found to have IC_{50} values of 0.11 μM and 0.11 μM respectively. However, *in vivo* activity was not observed. This was attributed to the poor aqueous solubility of the compounds, which will ultimately affect their bioavailability potential. A range of 7-amino-4-quinolone-3-carboxylic acids with alkyl, cycloalkyl and heterocyclic groups such as pyridine and pyrrole at the 7-amino position [140-142], as demonstrated by compound (**52**) have also been generated and screened against GP. These compounds were variable in inhibition of GP, and exhibited 4-100% enzyme inhibition at 10 μM.

Heterocyclic amides have also been reported as inhibitors of GP, primarily in the patent literature and often without specific inhibition data being supplied [117, 121, 122, 143-149]. The heterocyclic components have included bicyclic pyrrolyl amides [121], thienopyrrole amides [122], N-indenyl-4H-thieno[3,2-b]pyrrole-5-carboxamides [143-145], 3-phenyl-2-propenamide piperazines, such as (**53**) [146] and fused pyrrolylcarboxamides [147, 148], where inhibitory activity (IC_{50}) was measured values occurred in the range of 100 μM to 1 nM [144]. In the case of propenamide diazepine (**53**) (IC_{50} = 170 nM) the related series of compounds was not further progressed due to lack of cellular activity, possibly due to insufficient membrane permeability. 2-Oxo-1,2-dihydropyridin-3-yl amides have also been studied, and were synthesized using a facile aminolysis reaction. Evaluation of the inhibitory activity of these compounds against RMGP identified the dichlorobenzyl pyridone (**54**) (IC_{50} = 6.3 μM), as the most potent [150].

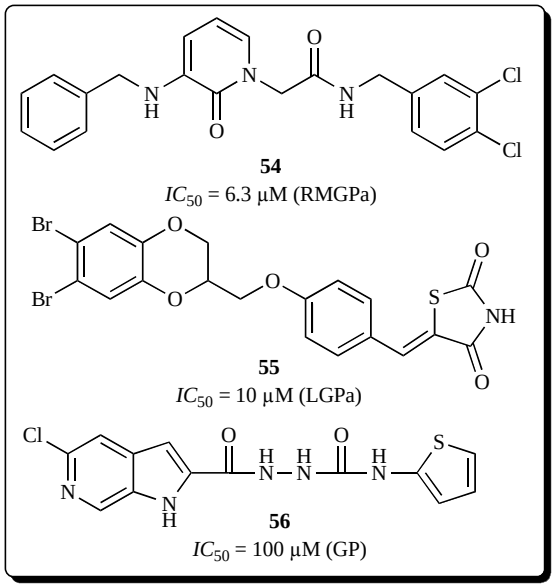


50; *n* = 0, R = 3-isoxazole IC_{50} = 0.11 μM (RMGPa)
51; *n* = 1, R = 2-thiophene IC_{50} = 0.11 μM (RMGPa)

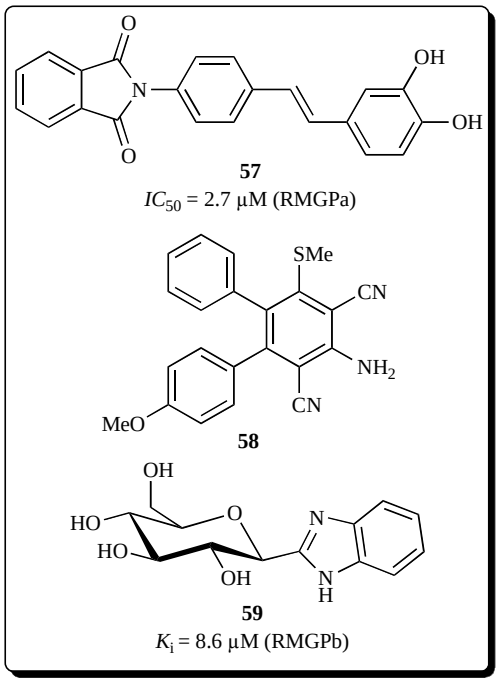
52

53
IC_{50} = 0.17 μM (HLGPa)

Heterocyclic scaffolds have included other heteroatoms in addition to nitrogen, such as sulphur and oxygen. For example, novel 5-benzyl and 5-benzylidene-thiazolidine-2,4-diones carrying 2,3-dihydrobenzo[1,4]dioxin pharmacophore have been synthesized and their GP inhibitor activity identified. 2,3-Dihydrobenzo[1,4]dioxin derivative (**55**) possessed a GP inhibitor activity with (Ki (GPa) = 10 μM) [151]. Other SAR studies of flavonoids revealed that the presence of the 3' and 4' OH groups in the B-ring and double bonds between C2 and C3 in flavones and flavonols are important factors for enzyme recognition and binding [152, 153]. Similarly, pyrrolopyridine-2-carboxylic acid hydrazides have been reported in the patent literature [107, 116] as having had IC_{50} values better than 100μM against GP. They have included (**56**), which was prepared from 5-chloro-1H-pyrrolo[2,3-c]pyridine-2-carboxylic acid hydrazide TFA salt and 2-thienyl isocyanate [107].



Relatively unfunctionalised and more complex aromatic compounds, when screened against GPa, display variable levels of inhibition. For example, gallic acid was determined to have an IC_{50} of 6.8 μM [154], whereas phenyl and benzyl substituted 2-oxo-hexahydro- and tetrahydrobenzo[cd]indole carboxylic acids had estimated IC_{50} values in the range of 0.8-1.3 mM [155]. Similarly, in the SAR studies of GPa inhibitors with a phenethylphenylphthalimide skeleton based on α-glucosidase inhibitors and liver X receptor (LXR) antagonists derived from thalidomide, the most potent compound was (**57**) (IC_{50} = 2.7 μM). This was approximately 40-fold more potent than a typical GPa inhibitor such as 1,4-dideoxy-1,4-imino-D-arabinitol (IC_{50} = 110 μM) [156]. Whereas, when the *in vitro* and *in vivo* antihyperglycaemic activity of 5,6-diarylanthranilo-1,3-dinitriles functionalised with donor–acceptor groups was reported, these compounds demonstrated good sugar lowering activity. Among various screened compounds, methylsulfanyl-[1,1';2',1'']terphenyl-3',5'-dicarbonitrile (**58**) showed 36.9% blood sugar lowering at 100 mg/kg dose in sucrose-challenged streptozotocin-induced diabetic Sprague–Dawley rat model [157]. The continued diversity in allosteric inhibitors is illustrated by compound (**59**). Recently, a possible novel binding site on the surface of GP located roughly 32 Å from any other binding area was reported. This site is mostly dominated by non-polar groups (Phe202, Tyr203, Val221 and Phe252) and binds 2-(β-D-glucopyranosyl)benzimidazole (**59**) [32], a β-D-glucopyranose analogue. It is interesting to note that some of the newer glucose derivatives bind to sites other than the catalytic site.

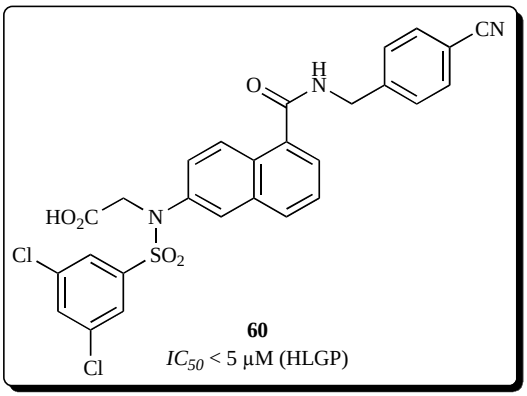


In addition to aromatic and heterocylic inhibitors, peptides have attracted recent interest. In particular, the peptide mastoparan has shown to be a GP inhibitor [158]. However, the majority of peptide work has targeted the interaction between GP and GL subunit of glycogen-associated protein phosphatase 1. Under glycogenolytic conditions, the interaction of hepatic glycogen-associated protein phosphatase-1 (PP1-GL) with GPa is believed to inhibit the dephosphorylation and activation of glycogen synthase (GS) by the PP1-GL complex, suppressing glycogen synthesis [159-165]. Consequently, an interaction of GL with GPa has emerged as an attractive target. The GL binding site on HLGPa is located 32 Å from the catalytic site, and 25 Å from the indole binding site at the subunit interface. This overlaps with the AMP binding site [165].

Studies on the C-terminal sequence of human GL that binds to GPa in the form of the synthetic peptide PEWPSYLGYEKLGPYY-NH_2, showed the peptide to display an IC_{50} of 34 μM against GPa [166]. By comparison, truncated peptides EKL-NH_2, EKLG-NH_2, and AcEKNH_2 inhibited GPa (21%, 32%, 63%, respectively at 22 mM) [166]. In other studies [163, 165] peptide $_{280}$LGPYY$_{284}$ comprising the last five amino acids of GL retained high-affinity interaction with GPa. The study demonstrated that the two tyrosines played crucial roles. Tyr284 deletion abolished binding of GPa to GL and replacement by phenylalanine was insufficient to restore high-affinity binding [163].

In a variation to peptide examples, substituted arylsulfonylglycines have also been tested as inhibitors of the inter-

action between GP and GL subunit of glycogen-associated protein phosphatase 1 [167-169]. In a binding test, arylsulfonylglycines, such as (**60**) inhibited the interaction of HLGP with protein PP1R3 (GL subunit of glycogen-associated PP1) with IC_{50} values < 5 μM being obtained [167-169].



60

IC50 < 5 μM (HLGP)

9. CONCLUDING REMARKS

As presented, considerable progress has been made in the identification of GP inhibitors over the past decade. A number of small molecule classes have been identified. The focus of work in the identification of inhibitors of GP has shifted from a primary interest into catalytic site inhibitors to a general interest in both allosteric and catalytic site inhibitors. During the last decade considerable progress has been made in matching the small-molecule classes of GP inhibitors with GP binding sites and in the development of allosteric GP inhibitors. The high degree of allosteric regulation of GP by a range of structurally diverse molecules opens up new possibilities for inhibitor design. At least one company is developing inhibitors of human GP for the treatment of type 2 diabetes mellitus (T2DM). GlaxoSmithKline has recently completed a Phase I clinical trial on the safety, tolerability, pharmacokinetics, and pharmacodynamics of GSK1362885 in Type 2 Diabetics. The results of this study are pending public release. However, the biggest obstacle for any GP inhibitor is the lack of isoform selectivity. This may partly account for why there are no GP inhibitors approved for the pharmaceutical market to date.

The development of GP inhibitors that might selectively inhibit liver and not muscle GPa remains a key issue. By avoiding inhibition of muscle GPa, impairment of the aerobic muscle function during prolonged contraction (state similar to McArdle's disease where mutation has lead to a deficiency of the muscle GP isoform) would be avoided [17, 18, 159]. Compounds developed to date do not display enough selectivity between liver and muscle GPa to avoid this undesirable side effect. Recent work has suggested that glycogen phosphorylase inhibition aimed at controlling blood glucose levels is unlikely to negatively impact on high intensity contraction of muscles and this may not be a clinical issue. However, the impact for prolonged low intensity contraction is unknown [17, 18]. Treatment of type 2 diabetes emphasises exercise and the clinical evaluation of nonselective inhibitors of GP will need to include muscle contraction endurance. Alternatively, the development of selective inhibitors for HLGP and/or other strategies for controlling excess glucose production in the liver should be the primary aim of work towards therapeutic agents for Type-2 diabetes. With an increasing structural diversity in identified GP inhibitors, and growing interest in research towards allosteric inhibitors may well need to be focussed towards the liver isoform of GP and the binding sites of GP where subtle differences in amino acid sequences lie (AMP-allosteric and indole binding site), as well as alternate allosteric regulation sites for GP, such as the PP1-GL binding site on GP. Thus control and regulation of GP in a selective and clinically effective manner may well be achieved in the future.

ACKNOWLEDGEMENTS

The author is grateful to Maria Petersson and David Karis for discussions during the preparation of parts of the manuscript.

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Received: March 26, 2010 Revised: July 01, 2010 Accepted: July 13, 2010