ISSN 1068-1620, Russian Journal of Bioorganic Chemistry, 2016, Vol. 42, No. 2, pp. 133–142. © Pleiades Publishing, Ltd., 2016.
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Glycogen Phosphorylase Inhibitors in the Regulation of Carbohydrate Metabolism in Type 2 Diabetes

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Received March 25, 2015; in final form, October 30, 2015

Abstract—Glycogen phosphorylase as a potential target for design of new drugs for the treatment of diabetes mellitus type 2, its main role and functions, as well as the ligands that bind to different sites of the enzyme are discussed in this review.

Keywords: type 2 diabetes, glycogen phosphorylase, glycogen phosphorylase inhibitors, catalytic site, caffeine binding (purine) site, site, allosteric AMP binding site, indole binding site

DOI: 10.1134/S1068162016020138

Type 2 diabetes (T2D) includes a heterogeneous group of carbohydrate metabolism disorders, which are based on insulin resistance, impaired secretory function of the pancreas and increased hepatic glucose production. In most patients with T2D the decrease in insulin sensitivity is a primary defect, while the inability of β-cells to maintain an adequate insulin secretory effect and hyperglycemia occur secondarily. It should be noted that in some patients, initial damage may occur at the level of β-cells and manifests as a disturbances of insulin secretion; insulin resistance develops together with or after the loss of insulin secretory function.

At present, the normalization of glycemia in diabetes is a rather complicated and problematic issue of diabetology. Despite the significant increase in the number of drugs and the occurrence of new pharmacological groups, it is difficult to achieve target blood glucose levels in patients with T2D [1]. A large number of oral antidiabetic drugs, which exert their effects through various mechanisms, aimed to eliminate three major metabolic disorders leading to hyperglycemia—dysfunction of β-cells, peripheral insulin resistance, excessive hepatic glucose production—are used in T2D therapy [2].

Drugs for the T2D treatment are represented by the following classes: *stimulators of insulin secretion*—sulfonylureas derivatives (glibenclamide, glipizide, gliclazide, gliquidone, glimepiride); *regulators of postprandial insulin secretion*—repaglinide and nateglinide, biguanidines—metformin; *insulin sensitizers*—rosiglitazone, pioglitazone; *incretin mimetics*—vildagliptin, sitagliptin, saxagliptin.

The presence of side effects and/or low efficiency of drugs used in the treatment of diabetes, promotes active research in this area [4]. It is advisable to start diabetes medication treatment with metformin according to the modern approaches, developed in collaboration with the European Association for the Study of Diabetes (EASD) and the American Diabetes Association (ADA). Metformin is the only drug that inhibits gluconeogenesis in the liver, whose the effect is associated with decreased hepatic insulin resistance. According to Long Y.C. (2006), metformin stimulates AMP-activated protein kinase and intracellular signal of energy depletion that leads to increased glucose uptake by skeletal muscle and inhibition of gluconeogenesis [5]. It should be noted that the increase in sensitivity of peripheral tissues to insulin by this drug is unstable. In addition, biguanides promote the increase in lactate, pyruvate, alanine levels in blood, which is associated with the disturbance of their clearance due to changes in the activity of mitochondrial pyruvate dehydrogenase complex, and as a result the development of lactic acidosis. The risk of lactic acidosis increases under kidney, liver and cardiovascular diseases, which limits the use of the drug [6].

Insulin resistance and relative or absolute insulin deficiency leads to glucose metabolism disorder in the liver followed by a decrease in the activity of the enzymes of *glycolysis* (oxidation of glucose to pyruvic acid) and *glycogen synthesis* (*glycogenesis*), and increased activity of the enzymes of *glycogenolysis* (breakdown of glycogen to glucose) and *gluconeogenesis* (glucose synthesis from noncarbohydrate sources). Promising direction for design of new drugs for the treatment of diabetes mellitus is the regulation of key carbohydrate metabolism enzymes: inhibition of glycogen phosphorylase (GP), phosphoenolpyruvate carboxylase, fructose bisphosphatase, glucose-6-

Abbreviations: GP, glycogen phosphorylase; HGP, hepatic glucose production.

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phosphatase and/or activation of glycogen synthase, hexokinase, phosphofructokinase, pyruvate kinase. Regulation of carbohydrate metabolism in the liver is the point for the application of new drugs for the treatment of diabetes mellitus, which are actively developed now.

Normally, the plasma glucose level is maintained within a narrow range during food intakes and between them. Glucose homeostasis is ensured by the balance between the absorption of glucose from the intestine and glucose uptake by peripheral tissues. It was proved that the liver plays a central role in glucose homeostasis [7]. This role lies in the ability of the liver to control hepatic glucose production (HGP): on the one hand, HGP is increased under fasting, and on the other, is suppressed during food intake, and glucose is stored in the liver as glycogen [8]. Thus, the control of HGP is one of the major regulators of glucose homeostasis.

HGP is determined by the balance between four main processes: *gluconeogenesis*, *glycogenolysis* and *glycolysis*, *glycogenesis*.

Between food intakes large part of glucose is supplied in the circulating blood by glycogenolysis [9]. Regulation of *glycogenolysis* is carried out together with the regulation of *glycogenesis* in the form of switching. This switching occurs at human organism transition from the absorptive state (postprandial) in postabsorptive state (between food intakes), as well as at the change of the state of rest to the state of physical work. In the liver it is performed under the participation of hormones insulin, glucagon and epinephrine, and in the muscle, insulin and epinephrine.

Breakdown of glycogen (Fig. 1) occurs via sequential removal of glucose residues in the form of glucose-1-phosphate [10]. The influence of hormones on the synthesis and breakdown of glycogen is performed by changes in activity of two key enzymes in opposite directions: glycogen synthase and GP by the means of their phosphorylation and dephosphorylation [11, 12]. In this way insulin inhibits glycogen phosphorylase and is a hormonal regulator of glucose homeostasis in the organism. Its relative or absolute deficiency or insulin resistance leads to an imbalance between supply of glucose and its utilization by peripheral tissues [13].

Gluconeogenesis becomes a source of glucose in the blood during fasting and depletion of glycogen stores [14]. This way along with glycogenolysis supports the blood glucose level required for processing of many tissues and organs, primarily, nervous tissue and erythrocytes.

In T2D, the synthesis of hepatic glucose from noncarbohydrate sources is activated due to a reduction in insulin levels, as well as tissue resistance to the insulin signal. Thus, the increase in hepatic glucose production is one of the main and driving factors in the pathogenesis of T2D and requires an additional pharmacological correction. It should be noted that a threefold or more increase in gluconeogenesis in the liver, observed under a moderate deficiency of insulin, is associated with the fact that a relatively greater amount of insulin is required for the suppression of gluconeogenesis than for the suppression of glycogenolysis.

The regulation of key enzymes of carbohydrate metabolism in the liver, as well as the inhibition of the enzymes of gluconeogenesis and glycogenolysis are the most important point of application for the compounds and the promising direction of finding new compounds for the treatment of T2D.

GLYCOGEN PHOSPHORYLASE

Glycogen phosphorylase (EC 2.4.1.1, EC 2.4) catalyzes phosphorolytic cleavage of the α-1,4-glycosidic bond in the glycogen molecule resulting in a cleavage of terminal nonreducing glucose residue with the formation of glucose-1-phosphate [10] and sequential breakdown of glycogen to glucose [15–17].

GP is a dimeric enzyme composed of two subunits. The enzyme can exist in two forms [18, 19]: GPa—the phosphorylated, catalytically active form; and GPb—the dephosphorylated, catalytically inactive form. In turn, each of the enzyme forms has the two conformations—active (R) and inactive (T) [20].

GP exists in a dynamic equilibrium between R- and T-state conformations, which is caused by two factors: phosphorylation/dephosphorylation of the enzyme and the interaction with an allosteric effector. The catalytic site in the T state is blocked by amino acid residues 282–286 (280s loop), and therefore GP cannot perform its biological function. GPb is activated by two mechanisms. Firstly, this is the phosphorylation of Ser14 in each enzyme monomer, which leads to a subsequent conversion into GPa. The R state of the enzyme is stabilized by phosphate groups of Ser14, and therefore GPa is preferably in the active R state. Secondly, the binding of allosteric effector, AMP with the allosteric site shifts the equilibrium from T to R state for the AMP enzyme. During this transition, the shift of the 280s loop occurs and the catalytic site opens. Thus, AMP binds to GPa and GPb, stabilizes the R state, enhances biological activity and affinity to the substrate.

It should be noted that glucose, glucose 6-phosphate, ATP and caffeine stabilize the T state and inhibit the enzyme [12, 15, 20]. Under certain conditions, GP can form tetramers. The position of dimer-tetramer equilibrium depends on GPb concentration, pH, ionic strength, temperature and the presence of allosteric effectors and substrates. Glucose 1-phosphate promotes association, while glycogen and inhibitors (ATP, ADP, flavins, etc.) prevents the formation of the tetrameric form. Under subunit-subunit contact, substantial changes in the quaternary structure of the protein, leading to the displacement of amino acid

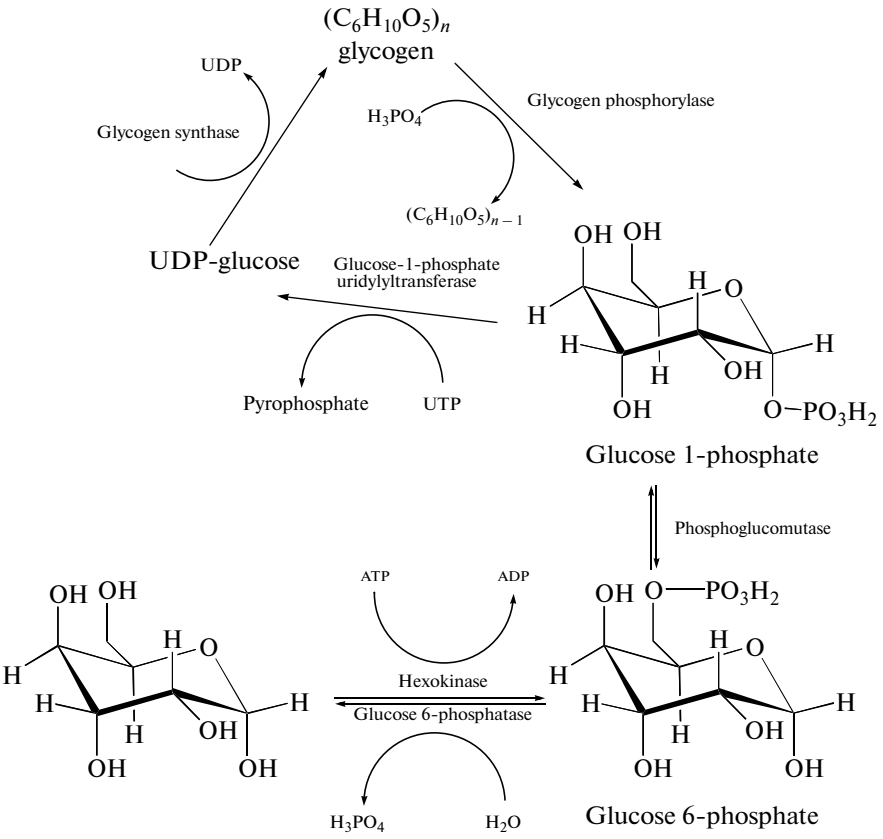


Fig. 1. Breakdown and synthesis of glycogen.

residues in the active site and the loss of GP biological activity, occur [20, 21].

The following sites for binding of physiological and pharmacological ligands have been found in the GP molecule as a result of X-ray analysis of rabbit muscle GP and human liver GP [22–25] (Fig. 2):

1. Catalytic site—the substrate is glycogen;

2. Allosteric AMP binding site;

3. Caffeine binding, inhibitory site (it binds purine nucleosides);

4. Glycogen storage site;

5. Allosteric indole carboxamide site;

6. Allosteric benzimidazole binding site.

The catalytic center is located in the center of the subunit and available for most of the ligands. Binding of a competitive inhibitor in this site promotes the formation of a less active T state via the stabilization of closed position of the 280s loop [26]. The allosteric site, which binds the activator AMP and the natural inhibitor glucose 6-phosphate is located on the surface of two subunits at a distance of about 30 Å from the catalytic site, and has recently attracted considerable interest from researchers [15].

The caffeine binding site is located on the surface the enzyme at a distance of 12 Å from the catalytic site and prevents the ligand entrance in the catalytic site in the inactive conformation. It is well described in the crystallographic studies, but little is known about its physiological role [27, 28].

The glycogen storage site is located on the surface of the molecule at a distance of about 30 Å from the catalytic site, and 40 Å from the allosteric AMP site. This site is not significant for the search for new drugs [29].

The indole carboxamide site, located inside the central cavity, is formed by coupling of two subunits. Depending on the binding site of the enzyme molecule, GP inhibitors are divided into: compounds which block the catalytic site and compounds which block the allosteric sites [30].

Catalytic Site Inhibitors

The following groups of chemical compounds were studied as inhibitors of the catalytic site of GP: *N*-acyl-β-D-glucopyranosylamine derivatives, *N*-β-D-glucopyranosyl oxanilic acid derivatives, *N*-β-D-glucopyranosyl ureas, 2-(D-glucopyranosyl)-5-methyl-1,3,4-oxadiazole derivatives, 2-(D-glucopyranosyl)-benzothiazoles, 2-(D-glucopyranosyl)-benzimidazoles, β-D-glucopyranosyl ammonium carbamate derivatives; *N*-β-D-glucopyranosyl monoamides of dicarboxylic acids; β-D-glucopyranosyl nucleosides;

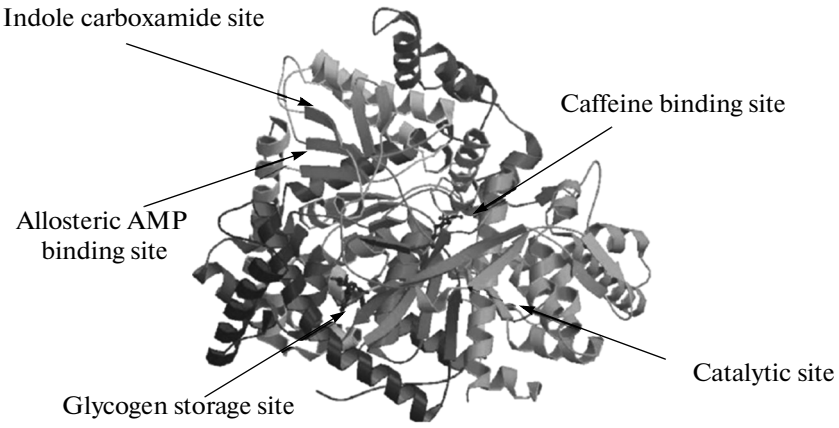


Fig. 2. Binding sites of GP.

3-substituted 5-β-D-glucopyranosyl-1,2,4-oxadiazoles; glucopyranosyliden-spiro-thiohydantoins; iminosugars [30–32].

Inhibitors of the catalytic site are represented by the compounds based on glucose due to the fact that glucose controls glycogen metabolism in two ways: by binding with the catalytic domain and stabilizing a less active T state of the enzyme, and also by dephosphorylation of active GP R state and enzyme inactivation [33].

A great number of glucose-based compounds that have a higher affinity to GP than glucose ($K_i = 2.0$ mM) was investigated as a result of numerous studies. The first attempts to develop a GP catalytic site inhibitor was associated with the synthesis of a series of compounds based on β-D-glucose (**I**) (Fig. 3) [33, 34]. The earliest studies of the compounds with GP inhibitory activity are devoted to *N*-acetyl-β-D-glucopyranosylamine (**II**) [35]. *N*- acetyl-β-D-glucopyranosylamine is a competitive inhibitor both for the b-form ($K_i = 32$ μM) and the a-form of the enzyme ($K_i = 35$ μM) [35–37]. Experiments on sedimentation rate determining have shown that *N*-acyl-β-D-glucopyranosylamine is able to induce dissociation of tetrameric phosphorylase a and to stabilize dimeric T state [36, 37].

Compound KV228, *N*-(3,5-dimethyl-benzoyl)-*N*-β-D-glucopyranosyl urea (**III**), is one of the most active inhibitor of the GP catalytic site. In animal experiments KB228 enhanced sensitivity of diabetic mice to glucose, which was accompanied by an increase in glucose uptake in the liver and increased glycogen deposition [37].

One of the potent inhibitors of the GP catalytic site is spirohydantoin of glucopyranose (hydan) (**IV**), whose inhibition constant is 550 times better than that of the natural ligand α-D-glucose [38]. Spirohydantoin analogues were also synthesized and examined for binding to GP [38–42].

Glucopyranosyliden-spiro-thiohydantoin (TH) represents the next class of inhibitors based on glucose with inhibition constant $K_i = 3–4$ μM. In vitro studies in liver extracts revealed that TH in the concentration of 50 μM enhanced activation of glycogen synthase, significantly reducing the time required for the dephosphorylation of the enzyme. In addition, TH reduced the activity of GP and increased phosphatase activity. TH restored glucose and glycogen levels to physiological values in the liver in streptozotocin induced diabetes [43].

Compounds of the class of iminosugars subdivided into mono- and bicyclic structures can be inhibitors of the catalytic site [44, 45]. 1,4-Dideoxy-1,4-imino-D-arabinitol (DAB), which is related to this class, is a potent inhibitor of phosphorylated and dephosphorylated GP forms [45, 46]. DAB inhibits glycogenolysis by direct inhibition of GP and does not affect protein phosphatase 1 (PP1), or kinases [47]. The study on the activity in vitro of this compound showed inhibition of GP in pig liver ($IC_{50} = 740 \pm 9$ nM) [15, 16]. Isofagomine (IFG) (**V**) (Fig. 4) and its *N*-substituted 3-phenylpropyl derivative (**VI**), that possess high activity, belong to this class of inhibitors [45].

Caffeine Binding (Purine) Site Inhibitors

Inhibitors of GP caffeine binding site are represented by the following groups of compounds: purines (uric acid, xanthine, hypoxanthine theophylline, allopurinol, 3-methylxanthine, 7-methylxanthine, 1-methylxanthine), flavopiridol and its analogues, vitamins (riboflavin, folic acid), indirubin (indirubin-3'-aminooxy-acetate (**IX**)) (Fig. 5) [48–50].

Ligand binding to this site occurs through the formation of π–π-bonds between the residue of an aromatic side chain Phe-285, the 280s loop and Tyr-613 [28]. Caffeine (**VII**) was the first inhibitor of purine site [28, 50]. Uric acid, xanthine, hypoxanthine, and allopurinol similarly to caffeine bind to the caffeine binding site, but shows a lower affinity than caffeine, whereas riboflavin exhibits a higher affinity. However, the physiological role played by these endogenous

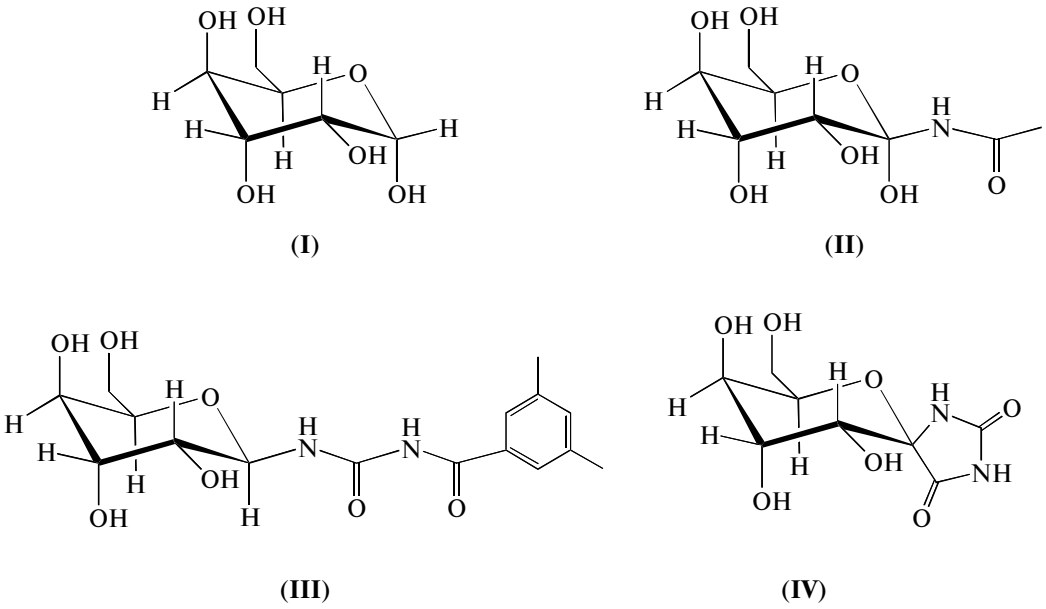


Fig. 3. Inhibitors of GP catalytic site: D-glucose (**I**), *N*-acetyl-β-D-glucopyranosylamine (**II**), *N*-(3,5-dimethyl-benzoyl)-*N*-β-D-glucopyranosyl urea (KB228) (**III**), spirohydantoin of β-D-glucopyranose (hydan) (**IV**).

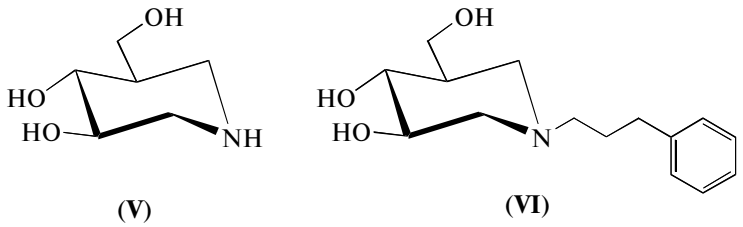


Fig. 4. Inhibitors of GP catalytic site: isofagomine (**V**), *N*-substituted 3-phenylpropyl isofagomine derivative (**VI**).

ligands in the regulation of hepatic glycogenolysis has not been elucidated [50].

Flavopiridol (**VIII**), a potential inhibitor of cyclin-dependent kinase with an antiproliferative effect on tumor cells [51], is another high affinity competitive inhibitor of hepatic GP and a ligand for the caffeine binding site [49, 52, 53]. This compound is used as a standard for determining the pharmacological activity of inhibitors of GP caffeine binding site. A series of analogs have been synthesized based on flavopiridol [12, 53].

Allosteric AMP Binding Site Inhibitors

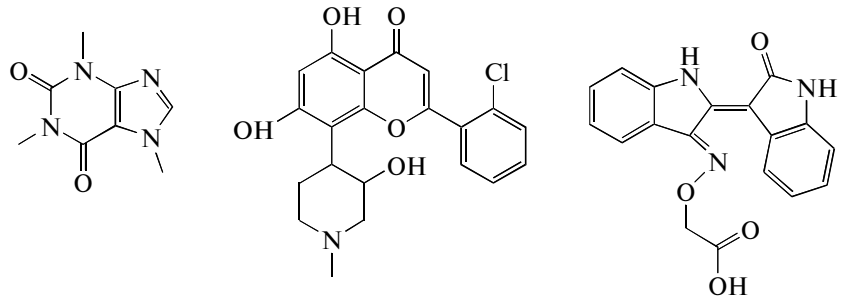
Ones of the first compounds, which were investigated as ligands for AMP binding site, are W1807 (**XI**) and prodrug R3401 (**XII**) [12, 54]. A number of compounds, that inhibit GP in nanomolar concentrations, was identified among the derivatives of dihydropyridine, benzoylphenylurea, acetylurea, phthalic acid [12, 29].

Compound W1807 (**XI**) (Fig. 6), several dihydropyridine analogues, phenyldicarboxylic acid analogues and phenoxyphthalates bind to this site and inhibit the basal and glucagon-induced glucose production in hepatocyte cell culture, resulting in a significant reduction in the peak of glucagon induced hyperglycemia when administered to rats [15, 29, 55].

Compound FR 258900 (**X**) binds to the allosteric AMP site and has an inhibitory effect on human hepatic GP. This compound significantly reduces plasma glucose concentrations under diabetes simulation in mice, and also stimulates glycogen synthesis and increases the activity of glycogen synthase in rat hepatocytes, IC_{50} = 2.5 μM (human liver GP), K_i = 0.46 μM (rabbit muscle GP) [46]. Thus, FR 258900 can be considered as a new potential antidiabetic drug. A number of active derivatives were synthesized based on FR 258900 [12, 29].

Indole Carboxamide Site Inhibitors

This group is represented by derivatives of indole-2-carboxamide (CP320626 (**XIV**), CP91149,



(VII) **(VIII)** **(IX)**

Fig. 5. Inhibitors of GP caffeine binding (purine) site: caffeine (**VII**), flavopiridol (**VIII**), indirubin-3-aminooxy-acetate (**IX**).

CP316819), indole-2-thienopyrrole carboxamide, *N*-aroyl phenylalaninamide, 5-chloroindole-2-carboxamide, and phenyl and benzyl substituted 2-oxo-tetrahydro- and hexahydrobenzo[*cd*]indole carboxylic acid [12, 29].

CP316819 (**XIV**) and its analog CP91149 (**XV**) are the most studied inhibitors of the indol binding site; they bind to the less active b-form of GP and prevent its transition to the active conformation [57, 58]. When analyzing the activity of the compound, it was shown that CP91149 has the structure similar to caffeine and inhibits glucagon secretion, but it is an inhibitor of the indole carboxamide site. In the experiment in mice with T2D this compound reduced the glucose level without causing hypoglycemia, and had no effect on the glucose level in intact animals [57].

It is proved that this class of GP inhibitors has a cardioprotective effect, which makes these compounds attractive for further preclinical studies in patients with cardiovascular complications [59].

The action of a GP inhibitor, GPi 688 (XVI), is aimed at the regulation of glucagon secretion, which causes hyperglycemia. In rat liver GPi 688 inhibits the effects of glucagon, resulting in decreased GP activity and sevenfold increased activity of glycogen synthase. In studies on the effect of the compound in rats of different types, GPi 688 inhibited the glucagon-induced hyperglycemia in Wistar rats (65%) and Zucker rats (100%) [60].

GP inhibitors reduce the breakdown of glycogen and HGP and affect one of the main pathogenetic factors of T2D; in addition to all these functions cardioprotective properties and antitumor effect also have been described [59, 61]. Patients with T2D have an increased risk of cardiovascular complications due to the suppression of fatty acid oxidation, glucose oxidation disorders, resulting in uncoupling of glycolysis in the cytoplasm and mitochondrial oxidative phosphorylation and as a result increase in the level of pyruvate and lactate in cardiomyocytes, calcium oversupply and disturbance of myocardium relaxation, which also increases the sensitivity of the myocardium to ischemic injury. Therefore, the question of the prevention of adverse effects of T2D arises [1, 9, 11].

Thus, cardioprotection is possible with the recovery of the conjugation of glucose oxidation and oxidative phosphorylation and reduction of anaerobic glycolysis [62]. Glycolysis becomes the main source of ATP under ischemia and infarction, and glycogen of cardiomyocytes is one of the main glycolytic substrates in a condition of limited coronary blood flow. But, under the condition of uncoordinated glucose oxidation in cytoplasm and oxidative phosphorylation in mitochondria, intense glycogenolysis leads to the accumulation of lactate and hydrogen protons and further development of the pathological process. A decrease in glycogenolysis via lowering the GP conversion into the active a-form is accompanied by inhibition of the development of pathological processes associated with the accumulation of lactate and hydrogen protons [63, 64]. As a result of GP inhibition, glycolysis is finally normalized and loading of ion channels is reduced; thereby, the cardioprotection without undesirable changes in cardiac function and hemodynamics is achieved [65–67].

This effect was confirmed by Tracey et al. [59]. The application of an indole-2-carboxamide derivative, CP368296 (ingliforib, 5-chloro-*N*-[(1S,2R)-3-[(3R,4S)-3,4-dihydroxy-1-pyrrolidinyl)]-2-hydroxy-3-oxo-1-(phenylmethyl)propyl]-1*H*-indole-2-carboxamide) reduced myocardial infarction size by 69% due to GP inhibition in vitro, as well as in vivo modeling of cardiac ischemia-reperfusion in rabbits. Furthermore, there were no significant changes in the function of the heart caused by the compound.

Thus, the GP inhibition may be an attractive target for cardioprotection, in the treatment of diabetic patients with an increased risk of cardiovascular complications.

The various observations show that the amount of glycogen differs in tumor cells of different origin. Its concentration is especially high in cancer cells of breast, kidney, uterus, bladder, skin and brain [68]. Recent studies have showed that glycogen plays an

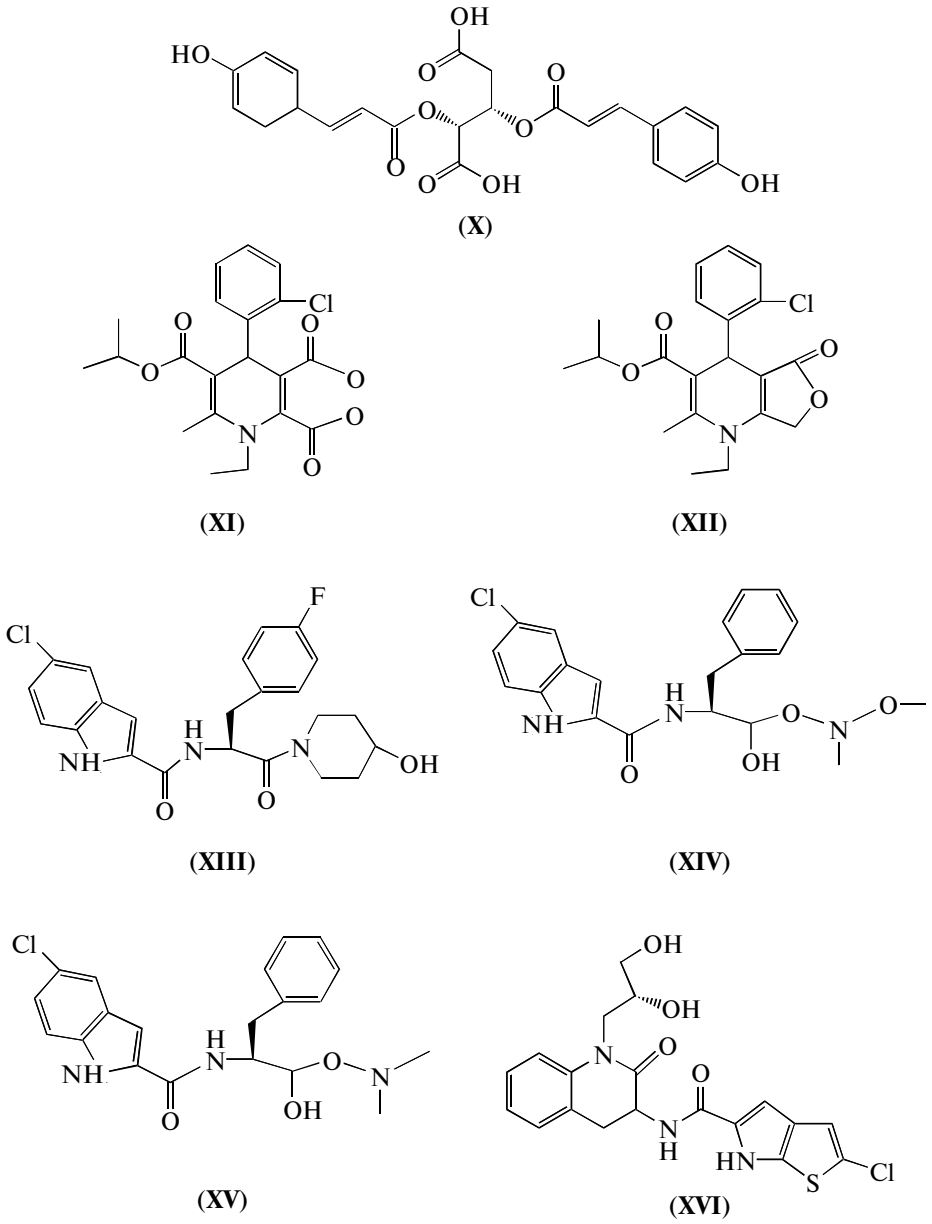


Fig. 6. Inhibitors of GP indole binding and AMP binding sites: FR 258900 (**X**), W1807 (**XI**), R 3401 (**XII**), CP320626 (**XIII**), CP316819 (**XIV**), CP91149 (**XV**), GPi 688 (**XVI**).

important role in the viability of cancer cells [69, 70] and the inhibition of its degradation leads to the induction of apoptosis in pancreatic cancer cells [69–71].

An increase in the number of cancer cells occurs due to active synthesis of RNA, DNA, and fatty acids, which in turn causes an increased need for glucose. A cancer cell during the S phase of its cell cycle requires the rapid consumption of intracellular glucose storage, which is regulated by glycogen phosphorylase [61, 70]. Hence, one can control the cell cycle of cancer cells by influencing the GP activity.

It also has been proved that glycogen synthesis is induced under hypoxia, that increases the survival of cancer cells. Glycogen breakdown via the GP inhibition provides a source of glucose 6-phosphate and limits the intracellular glycogen level [66, 67, 70].

Lee N. P. et al. [61] demonstrated that the GP inhibition slows the breakdown of glycogen, and also inhibits cancer cell proliferation and induces apoptosis. Higher concentrations (50–100 mM) of GP inhibitor CP320676 changed the redistribution of glucose and pentose between glycolysis and fatty acid synthesis in cells of pancreatic adenocarcinoma. Limiting the oxidative pentose phosphate pathway and contribution of glucose in the synthesis of acetyl-CoA and fatty acids de novo closely correlated with decreased cell

proliferation [61]. In conclusion, it should be noted that the inhibition of pancreatic cancer cell growth and their death are closely related to the characteristic decrease in glycogen breakdown and redistribution of glucose carbon for the synthesis of RNA/DNA and fatty acids under incubation with the GP inhibitor. Significant dose-dependent decrease in the synthesis of macromolecules that were detected at lower doses of the test compound, before the markers of apoptosis appeared, was established when evaluating the dynamics of cancer cell metabolism.

Thus, the slowdown of glycogen synthesis via the GP inhibition can be successfully used for development of the drugs that effectively reduce the growth of rapidly dividing cancer cells.

CONCLUSIONS

Inhibition of glycogenolysis is one of the perspective pharmacological approaches for potential T2D therapy. GP inhibitors reduce the breakdown of glycogen and HGP, and affect one of the main pathogenetic factors. HGP is determined by the balance between the four main processes: gluconeogenesis, glycogenolysis and glycolysis, glycogenesis. The decrease in the GP activity significantly attenuates hyperglycemia as well as does not lead to hypoglycemia, which is one of the most important aspects in the glucose-lowering therapy. All the aforesaid confirms the relevance and the prospects of finding new compounds that inhibit GP.

ACKNOWLEDGMENTS

This work was supported by the Russian Science Foundation (project no. 14-25-00139).

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Translated by D. Novikova