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New target for glycogen phosphorylase inhibitors: sodium-glucose co-transporter of the kidney

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Introduction

Diabetes mellitus (DM) is one of the most prevalent diseases on earth. The number of adults with diabetes has more than tripled in the last 20 years, so diabetes is one of the fastest growing health challenges of the 21st century. The rise in the number of people with diabetes is driven by a combination of socio-economic, demographic, environmental, and genetic factors. Diabetes is a serious, long-term (chronic) disease that occurs when blood glucose concentrations are elevated. Glucose elevations are caused by the inability to produce enough insulin or the lack of effective responses to insulin. Insulin is a peptide hormone produced by beta cells in the islets of Langerhans in the pancreas, which allows glucose circulating in the bloodstream to enter the cells of the body where it is converted into energy. Insulin deficiency, if left untreated over a long period, can lead to multiple organ damage and life-threatening health complications, such as retinopathy, blindness, kidney failure, cardiovascular disease, neuropathy, stroke, and amputation of a finger, foot, or leg. If diabetes is managed properly, these serious complications can be delayed or prevented altogether.

There are two main types of diabetes, type 1 and type 2. Type 1 diabetes is caused by an autoimmune reaction towards insulin-producing beta cells in the pancreas. As a result, the body produces little or no insulin. People with type 1 diabetes need daily insulin injections to maintain adequate blood glucose levels. With proper daily insulin management, regular blood glucose monitoring, support, and education, patients can lead healthy lives and delay and prevent complications associated with diabetes. Type 1 diabetes can develop at any age, but is most commonly discovered in children and young people. Typical symptoms include excessive thirst (polydipsia), frequent urination (polyuria), fatigue, constant hunger, sudden weight loss, bedwetting, and blurred vision.

In type 2 diabetes, the body's cells are unable to respond fully to insulin, leaving more glucose in the blood, a condition known as insulin resistance. In this condition, the effectiveness of the hormone decreases and insulin production increases. Over time, the beta cells in the pancreas cannot keep up with demand and inadequate insulin production develops. Type 2 diabetes is most common in older adults. However, due to increasing obesity, physical inactivity and inadequate diet, type 2 diabetes is also increasing in children and younger adults. Type 2 diabetes has similar symptoms to type 1 diabetes, but the onset is less dramatic. An important part of managing type 2 diabetes is a healthy lifestyle, including regular exercise, a healthy diet, no smoking, and maintaining a healthy weight. If attempts at lifestyle changes are not enough

to control blood glucose levels, oral medication is needed. If oral antidiabetics are not able to control blood glucose levels adequately, insulin injections may be needed.

Glycogen metabolism

The majority of glucose is used aerobically by the brain. Most of the remainder is utilized by red blood cells, skeletal muscle, and myocardium. Our body can obtain glucose directly from the diet or from amino acids and lactate through gluconeogenesis. Glucose obtained from these two primary sources either circulates in the bloodstream or is stored by the body in a polymer form, called glycogen.

Glycogen is a branched-chain glucose polymer linked by α-(1,4)- and α-(1,6)-glycosidic bonds at the branches. In cells, glycogen is found in glycosomes, which are protein and glycogen complexes. Most glycogen is stored in the liver and muscles, but the brain, uterus, and vagina can also synthesize glycogen. The liver is the only tissue capable of releasing glucose from glycogen into the bloodstream, thus regulating blood glucose levels. The liver's glycogen stores are the main buffer for blood glucose. Other tissues can also synthesise and break down glycogen, but they use this glucose only to cover the energy needs of their cells. Glycogen homeostasis involves the coordinated regulation of the rate of glycogen synthesis (glycogenesis) and the rate of glycogen breakdown (glycogenolysis). The two processes operate by reciprocal regulation. Hormones that stimulate glycogenolysis (glucagon, cortisol, adrenaline, noradrenaline) simultaneously inhibit glycogenesis. In contrast, insulin, which stimulates glycogenesis, inhibits glycogenolysis.

The key enzyme for glycogen synthesis is glycogen synthase (GS). GS can form α-1,4 bonds using UDP-glucose as a substrate. Two different isoforms exist in humans. One is dominant in skeletal muscle and the other is dominant in the liver. The liver isoform contains 7 phosphorylation sites and can be regulated by enzyme synthesis, phosphorylation, dephosphorylation, and allosteric regulation. In our organism, the main regulator is glucose-1-phosphate. The key enzyme for glycogen degradation is glycogen phosphorylase (GF), whose activity is regulated by its phosphorylation state. The function of phosphorylase is to phosphorylate glucose by cleaving the α-(1,4) bonds of glycogen. The product of the reaction is glucose-1-phosphate and one less glucose number of glycogen molecules. Under normal conditions it has a dimeric structure. There are 3 isoforms of GF: liver, muscle, and brain specific enzymes. GF plays an important role in the regulation of blood glucose levels, which is why several research groups have designed compounds that can lower blood glucose levels by inhibiting the enzyme. X-ray crystallography studies of the dimeric enzyme have identified

5 ligand binding sites through which specifically designed molecules can inhibit the activity of the enzyme, namely the active site, nucleotide binding site, AMP binding site, indole binding site, and glycogen binding site. The activities of kinases involved in the regulation of glycogen metabolism are mainly controlled by cAMP and Ca^{2+}. Glucagon stimulates phosphorylation of GF via cAMP. Glucagon activates adenylate cyclase, which produces cAMP. The cAMP activates cAMP-dependent protein kinase, which phosphorylates phosphorylase kinase. The latter in turn phosphorylates GF, generating GFa and triggering glycogen degradation. Increased Ca^{2+} concentration stimulates glycogen degradation by increasing the activity of phosphorylase kinase. Vasopressin, adrenaline, oxytocin, and angiotensin 2 also stimulate glycogen degradation through activity of phosphorylase kinase. Phosphatases can also affect glycogen degradation by dephosphorylating GFa and activating GS, triggering glycogen synthesis.

Glucose transporters

In mammalian cells, glucose is transported by glucose transporters located in the cell membrane. Structurally and functionally, the transporters can be divided into 2 types: glucose transporters (GLUT), which operate by facilitated diffusion, and sodium-glucose co-transporters (SGLT), which transport glucose by active transport against a concentration gradient by sodium binding. GLUTs make the glucose concentrations on both sides of the plasma membrane identical because they are driven by a glucose gradient, but SGLTs can create 2 different glucose concentrations between the 2 sides of the membrane because they are driven by a sodium gradient for glucose uptake.

SGLTs are a large family of membrane proteins. Six human isoforms of SGLTs are distinguished. Glucose and sodium are transported into cells together against a sodium concentration gradient. Of these, SGLT 1 and SGLT2 are the best known, as they play key roles in the transport of glucose and sodium across the membrane of intestinal and renal epithelial cells. SGLT1 is mainly responsible for glucose absorption in the small intestine, while SGLT2 is responsible for glucose reabsorption in the kidney. Considering the physiological functions of SGLT1 and SGLT2, they have become reasonable targets for drug discovery. In 1987, phlorizin, a naturally occurring inhibitor of SGLT1 and SGLT2, was reported in a study to reduce the symptoms of diabetes in rats undergoing pancreatectomy. Subsequently, SGLT2 inhibitors based on the structure of phlorizin have been developed and compounds have been identified and licensed.

Human SGLT2 has K_M values of 2 mM for glucose and 25 mM for sodium. Unlike SGLT1, SGLT2 is a low affinity, high capacity glucose transporter. In the kidney, SGLT2 and SGLT1 transport glucose through the apical membrane of the proximal convoluted tubule to the cell interior and glucose exits the cell through the basolateral membrane via GLUT2 and GLUT1. In humans and rodents, SGLT2 is expressed in the upper part of the proximal tubule (S1 and S2 segments), while SGLT1 is expressed in the lower part of the proximal tubule (S3 segment). Under euglycaemia (arterial blood glucose concentration 5.5 ± 0.5 mmol/l), 80% or more of the reabsorption of filtered glucose is performed by SGLT2, while SGLT1 is responsible for the reabsorption of the remaining glucose. In addition to the former, the ratio of glucose to sodium also differs between the two transporters: SGLT2 transports glucose and sodium in a 1:1 ratio, whereas SGLT1 transports glucose and sodium in a 1:2 ratio. When pharmacological inhibition of SGLT2 is induced, the amount of glucose increases in the distal part of the proximal tubule, and SGLT1 partially compensates for glucose reabsorption. Therefore, the fractional glucose reabsorption of euglycaemic people treated with SGLT2 inhibitors is reduced to only 40-50%.

SGLT2 inhibitors

Current hyperglycaemic agents target pathophysiological defects in diabetes, appetite control, nutrient absorption, and excretion. Hyperglycaemic agents lower blood glucose levels by a number of different mechanisms. Metformin inhibits hepatic gluconeogenesis. Sulphonylurea derivatives and glucagon-like peptide 1 (GLP-1) analogues and dipeptidyl peptide 4 (DPP) inhibitors increase insulin secretion in the pancreas. Thiazolidinediones increase insulin sensitivity. However, blood glucose levels are often difficult to maintain within the optimal range of oral antidiabetic agents. If a single agent fails to achieve adequate results, combination therapy is recommended, using several drugs with different mechanisms of action. Despite the use of hypoglycaemic drugs, glycaemic control in diabetic patients remains a problem and new drugs need to be developed to reduce mortality and complications and improve quality of life. In addition to combination therapies, a new approach to drug design is the search for dual or multi-target compounds that can act on multiple biological macromolecules. The development of such multi-target drugs in the context of type 2 diabetes has recently been reviewed. The newest antidiabetic drugs on the market are sodium-dependent glucose cotransporter 2 (SGLT2) inhibitors, known as gliflozins. Gliflozins reduce renal absorption of glucose, increasing the concentration of glucose in the excreted urine, thus

lowering blood glucose levels in diabetic patients. There are currently seven marketed SGLT2 inhibitors available as oral antidiabetic agents for use in type 2 diabetes.

Gliflozins are used alone or in combination with other oral antidiabetic medicines. SGLT2 inhibitors also inhibit SGLT1 and have different selectivities for SGLT1 and SGLT2 and. The SGLT2/SGLT1 selectivity is 1000-fold or higher for dapagliflozin, empagliflozin, luseogliflozin, ertugliflozin and tofogliflozin, while the selectivity of canagliflozin and ipragliflozin is 190- and 250-fold, respectively. Sotagliflozin, a dual inhibitor of SGLT2 and SGLT1, has been approved by the EMA but not by the FDA as an adjunct to insulin in type 1 diabetes. Sotagliflozin has dual actions, blunting and delaying the absorption of glucose from the gastrointestinal tract and the reabsorption of glucose in the renal proximal tubule. In preclinical studies in diabetic animal models, SGLT2 inhibitors have reduced fasting and non-fasting blood glucose levels, HbA1c levels, and blood pressure, and improved glucose intolerance. Meta-analyses with SGLT2 inhibitors have shown that death and hospitalisation time and death from any cause were reduced among people with type 2 diabetes and cardiovascular disease treated with gliflozins, when the study drug was added to usual care. Recent studies have reported that SGLT2 inhibitors have a kidney-protective effect in diabetic nephropathy animal models, and the kidney-protective effect has also been demonstrated in clinical trials.

Patients taking glyphosate may rarely develop diabetic ketoacidosis, a serious complication of diabetes that can be life-threatening. In some cases, ketoacidosis developed because the patients' blood glucose levels were not as high as expected.

Chemically, the already approved gliflozins are C-glucopyranosylmethyl(het)arenes. In order to improve the pharmacodynamic and pharmacokinetic properties of phlorizin, a number of structure-activity relationships were investigated by modifications of the basic scaffold of the compound. This resulted in a number of O- and N-glycosidic compounds, of which the C-glycosyl structure was finally found to be the most suitable. After accidentally discovering the highly preferential meta location of glycosyl and benzyl groups on the proximal aromatic ring, this scaffold became the lead structure for further structure-activity relationship experiments. The effects of the newly synthesised molecules were investigated by modifying the sugar moiety, the aromatic rings, and the methylene bridge between them. While the replacement of the distal aromatic group has been extensively investigated, resulting in the already commercialised kanagliflozin and ipragliflozin, far fewer studies have addressed the replacement of the proximal benzene unit with a heterocyclic compound. Known structures include thiophene, pyrrole, thiazole, pyridine, pyridazine, and pyrazine rings with IC_{50} values

ranging from low nM to low μM. IC_{50} values are strongly dependent on the heterocyclic group. The introduction of the phenolic -OH group in the ortho position on the proximal ring has been shown to be advantageous in several cases, highlighting the potential role of the hydrogen bond-forming group in this region. Further substitution of the proximal benzene ring was planned as the heterocycle may have a significant impact on the efficiency of the molecules. It is anticipated that imidazole and 1,2,4-triazole derivatives may form H-bonds close to the -OH position of the former phenol. Furthermore, considering that the glucosylbiphenyl derivative inhibits SGLTs, these compounds will be studied as well. In addition, many derivatives with similar structures are also effective inhibitors of glycogen phosphorylase, which have previously been identified as another target in the battle against type 2 diabetes. These compounds may possess SGLT-GP dual inhibitory activity, which has not been previously investigated.

Aims

Diabetes has become widespread and the number of patients is increasing. The most common symptom of diabetes is hyperglycaemia. Better blood glucose level reduction is one of the most important goals of treating the disease. A new target of oral hypoglycaemic medication to decrease blood glucose is inhibition of the sodium-glucose cotransporter 2 (SGLT2). The general structure of the SGLT2 inhibitors based on a summary article by Sebastian Vidal contains the glucopiranose ring, a C-glucosidic bond with a ring, and one more aromatic group connected by a methylene bridge.

For over 20 years, our research group has been identifying glycogen phosphorylase (GP) inhibitors, but these compounds are not clinically used drugs. Several glucose analogue inhibitors were synthesized by our collaborators. The first aromatic ring is a heteroatomatic ring, such as pyrimidine, oxadiazole and triazole instead of benzene, in these compounds.

Therefore, the aims of my work were to:

- test more potential GP inhibitors
- test more potential SGLT 1 and 2 inhibitors in a cell system
- create new cell lines which overexpress the SGLT1 and 2 proteins for testing the new compounds
- review previously identified GF inhibitor compounds with similarities in structure to the general structure of SGLT2 inhibitors, to see which compounds can inhibit SGLT2 in the cell system

Methods

Skeletal muscle glycogen phosphorylase b enzyme preparation from rabbit skeletal muscle

Homogeneous GFb was prepared from rabbit skeletal muscle according to Fischer and Krebs, recrystallized three times, and the crystals were dissolved in TEM buffer (40 mM Tris-HCl, 2 mM EDTA ,10 mM ME, pH = 6.8). Nucleotides (primarily AMP) were removed by Norit A cellulose treatment. The specific enzyme activity of the preparations was 48–55 U/mg in the presence of 16 mM G-1-P and 1 mM AMP. Homogeneity was checked by SDS-polyacrylamide gel electrophoresis and a single staining of a 95 kDa protein was observed.

Determination of purified muscle glycogen phosphorylase activity

Kinetic measurements with purified glycogen phosphorylases were performed according to a method described in the literature. Enzyme activity was measured in the direction of glycogen synthesis (in contrast to the in vivo process). Skeletal muscle and liver GFα activity were determined in the presence of different concentrations (2–16 mM) of G-1-P and constant concentrations (1%) of glycogen in 50 mM triethanolamine, 100 mM KCl, 1 mM EDTA (pH 6.8) in the presence of a buffer. For skeletal muscle GFb, the incubation mixture also contained 1 mM AMP (as an activator). The activity was determined at 30°C and the incubation time was adjusted so that up to 20% of the substrate (G-1-P) was converted. The amount of GF that catalyzes the conversion of 1 μmol of phosphate in one minute at 30°C was considered a unit.

Investigation of glycogen phosphorylase inhibitors: Determination of K_i and IC_{50}

Dixon plotting was used to determine the inhibition constants of the test compounds (potential GF inhibitors). Our hypothesis was that molecules synthesized from glucose bind to GF following Michaelis-Menten kinetics. The Michaelis-Menten equation is a hyperbola from which the maximum rate of enzyme reaction (V_{max}) and the Michaelis-Menten (K_M) constant can be determined. One way to graphically determine constants is to linearize the original equation using the Lineweaver-Burk representation. This linearization is based on taking the reciprocal of the original equation, and the axial sections of the resulting straight line can be used to determine the enzyme reaction constants more accurately. When the rate of the enzyme reaction was measured at several different inhibitor and substrate concentrations, more lines were obtained in the linearized Lineweaver-Burk plot according to the different concentrations of inhibitor. Plotting the slope of the lines thus obtained as a function of the corresponding inhibitor concentration gives a new curve whose x-axis section gives the inhibition constant

(K_i). Inhibition constants were determined by computer evaluation of the measurement data (GraphPad Prism).

For compounds that do not inhibit the GF enzyme even at high concentrations, determination of the inhibitor concentration (IC_{50}) required 50% inhibition of the maximally achievable activity, as the common method. The essence of the measurement is the same as the determination of K_i, the difference being that the IC_{50} is determined by varying the concentration of inhibitor at a single substrate concentration and the concentration of inhibitor required for a 50% reaction rate is called the IC_{50}.

Cell culture

TSA201 (human embryonic kidney) cells were maintained in 25 mM glucose medium containing 10% FBS and 1.5% L-glutamine. The medium of the overexpressed cell lines also contained 10 μg/ml puromycin. Cells were maintained in T-75 cell culture flasks at 37°C in a 5% CO_2 incubator. Trypsin was used for passage and the cells were washed with phosphate buffer.

Lentiviral transfection

TSA201 cells (100,000 per well) were plated in 24-well plates and kept in an incubator at 37°C for 24 hours. The next day, the media of the cells were replaced containing media containing 8 μg/ml hexadimethrin bromide, and the corresponding wells containing the lentiviral vector. After 24 hours, the cell culture medium was changed to fresh medium. After 3 days, the cells were harvested and transferred to T-25 culture flasks. On day 5, 10 μg/ml puromycin was added to the cell culture medium, which was subsequently added to the fresh cell medium every time. Thereafter, the cell medium was changed to fresh medium every 2 days until selection was complete relative to control cells.

Western blot

Cells were cultured in 60 mm petri dishes. Cells were washed with phosphate buffer and collected on ice with 100 μl TET lysis buffer. The lysate was incubated on ice for 45 minutes and vortexed 3 times and then stored at -80°C. After thawing the lysates, the protein content was measured and the samples were boiled for 10 minutes at 100°C after adding sample buffer. Proteins in the sample were separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Samples were loaded on 4–16% gradient gel. Electrophoresis was run at 80 volt in Tris buffered saline buffer. After gel electrophoresis, proteins were transferred from the gel to a PVDF (polyvinylidene fluoride) membrane in transfer buffer at constant 0.35 amps

for 60 min. The membrane was blocked with 5% BSA (bovine serum albumin) solution for 1 h to prevent nonspecific antibody binding. After blocking, membranes were incubated overnight at 4°C with solutions of SGLT1, SGLT2, and GAPDH antibodies in 1% BSA. The next day, the membranes were washed 3 times with TBST solution, and incubated in the appropriate secondary antibody (dissolved in TBST and 1% BSA) solution for 1 hour. After incubation, membranes were washed twice with TBST solution and once with TBS solution. Secondary antibodies were conjugated with horseradish peroxidase, so antibody binding to specific proteins was detected with a solution containing chemiluminescent substrate. The results were documented using the ChemiDoc Touch Imaging System. Data were analysed using Image Lab software.

Protein measurement

The bicinchoninic acid (BCA) method was used to determine protein concentration. The experiments were performed in a 96-well plate. Samples (25 µl) were added to each well, to which 100 µl of BCA reagent was added. Samples were incubated for 30 minutes at 37°C, and absorbance was measured at 562 nm on a photometer.

Glucose uptake measurement

TSA201 cells (25,000 per well) with and without overexpression of SGLT1 and SGLT2 were plated on 96-well plates pretreated with poly-L-lysine. The next day, the medium of the cells was changed to sugar-free medium containing FBS, L-glutamine, and the compound to be tested. After starvation, 100 µM 2-NBDG was added to the cell medium and incubated for half an hour. After incubation, the supernatant was removed, cells were washed twice with phosphate buffer, KH_2PO_4 lysis buffer was added, and samples were stored at -20°C. After thawing, the samples were suspended and the fluorescent signal was measured using a fluorimeter (excitation wavelength: 485 nm, emission wavelength: 538 nm). The protein content of the samples was then measured to normalize the glucose uptake of the cells.

SGLT1 and SGLT2 IC_{50} calculation

After selecting the optimal concentration range, each compound was tested at 4 or more different concentrations (0.1 and 1000 µM). Glucose uptake values for a cell line without overexpression were subtracted from the values for an overexpressed cell line, so subtracting from the overexpressed control yielded 100% of the original control. GraphPad Prism software was used for all calculations. Data were plotted on a logarithmic scale as a function of inhibitor

concentration. IC_{50} values were calculated by non-linear regression analysis from sigmoidal dose-response curves.

Cytotoxicity measurement

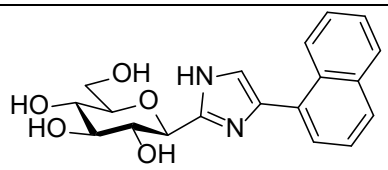
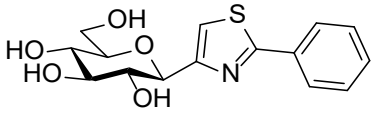
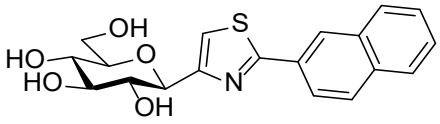
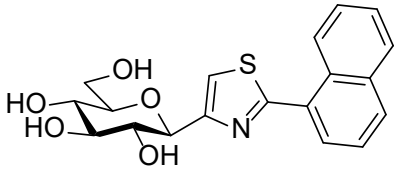
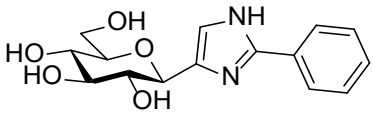
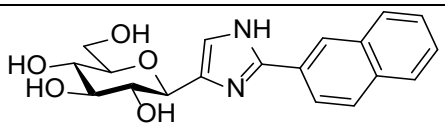
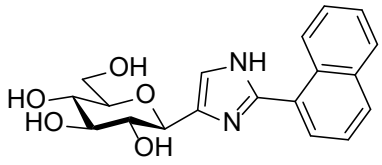
TSA201 cells were used for cytotoxicity measurements, and the viability of cells was measured using a fluorescent CyQUANT assay. TSA201 cells (25,000 per well) were placed in a 96-well plate. The next day, the cell supernatant was removed and replaced with fresh complete medium containing the compounds to be tested (1 mM) and doxorubicin (2 ng/ml) as a negative control. Positive control contained DMSO only. After 4 hours of incubation, the supernatant was removed and the cells were washed with phosphate buffer and the plates were frozen. The next day, the plates were thawed and 200 µl of CyQUANT reagent was added to the wells. Protected from light, after 5 minutes of incubation at room temperature, the fluorescent signal was measured with a fluorimeter (excitation wavelength: 480 nm, emission wavelength: 520 nm). The values obtained are plotted against the control.

Results and Discussion

Structure - inhibition studies of C-β-D-glucopyranosyl azole compounds

The scientific progress of the last decades has opened new opportunities for research on new potential drug molecules. The use of computer technology has led to structure and ligand based design. Recognizing the structural relationships of previously studied compounds, Joseph M. Hayes and colleagues have used computer simulation to model new C-β-D-glucopyranosyl azole compounds that had not previously been synthesized and studied as GF inhibitors. In the simulation, the compounds were ranked according to their binding strength to the active site. Seven compounds were selected from the modelled molecules and synthesised by the group of Dr. László Somsák at the Department of Organic Chemistry, University of Debrecen. We performed the measurement and analysis of the prepared compounds on rabbit skeletal muscle glycogen phosphorylase b and compared the simulated values with the in vitro experimental results.

Table 1. Structure of 7 selected C-β-D-glucopyranosyl heterocycles

Code	Structure	Code	Structure
5c		**12a**	
12b		**12c**	
13a		**13b**	
13c			

Similar to the previously studied glucose derivative inhibitors, the compounds we investigated were competitive inhibitors, as evidenced by the Dixon diagrams of the

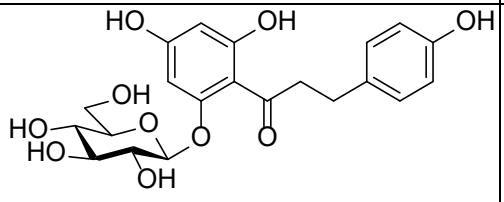
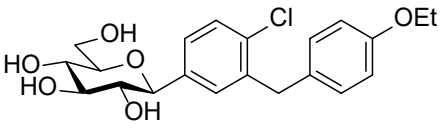
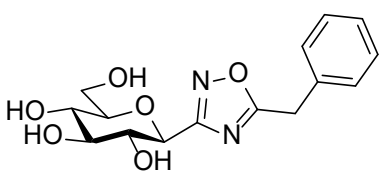
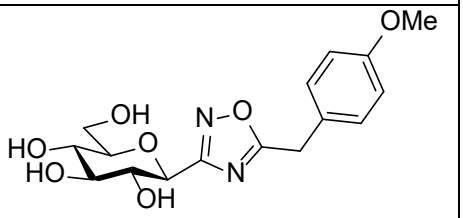
compounds, since the intersection points of the lines of their diagrams are in the negative region of the x-axis, which is a characteristic of competitive inhibition.

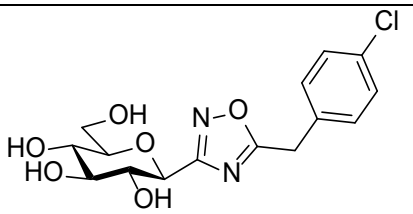
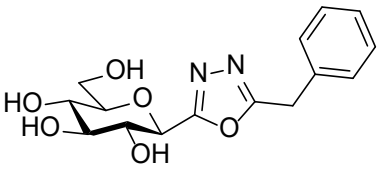
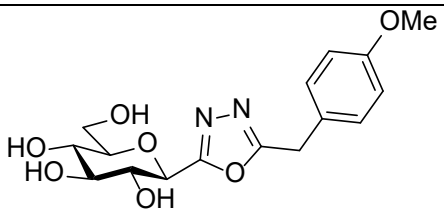
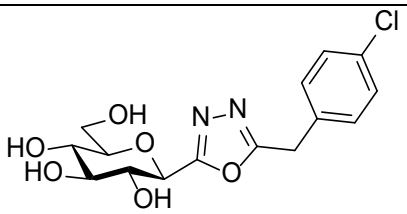
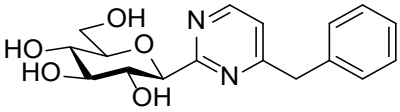
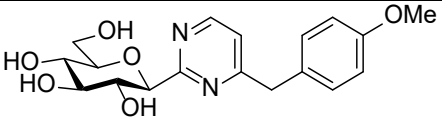
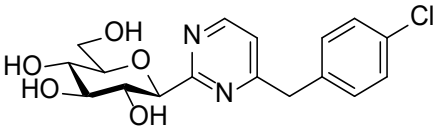
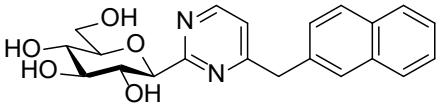
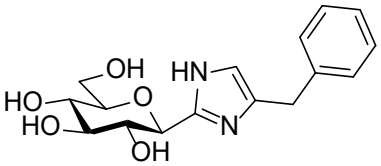
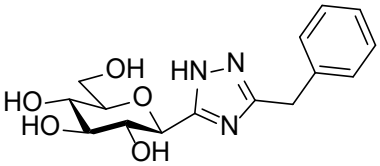
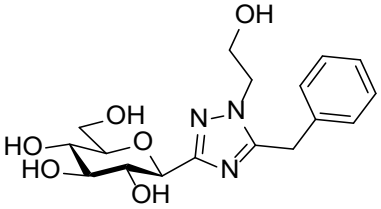
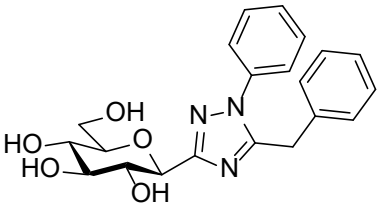
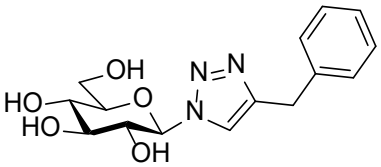
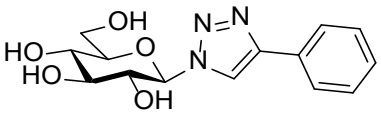
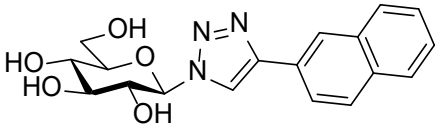
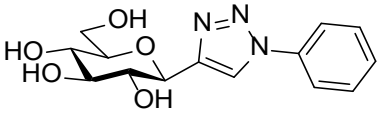
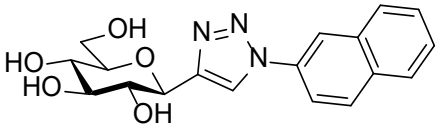
Of the compounds tested, compound 5c, predicted to be the most potent, was able to inhibit GFb the most strongly (K_i = 1.97 μM), but its inhibitory effect was below the nanomolar inhibition values of the analogues tested previously. The inhibitory ability of the imidazole derivative 13b (K_i = 4.58 μM) was as calculated, but the calculated values of 13a (K_i = 68.6 μM) and 13c (K_i = 71.1 μM) were lower than the measured values, the latter discrepancies being due to the settings of the simulation model set. Among the thiazole derivatives, the measured value of 12b (K_i = 26.2 μM) was similar to the simulation value, but the phenyl derivative 12a (K_i = 326 μM) and the 1-naphthyl derivative 12c (K_i = 540 μM) compounds were much weaker than expected in inhibiting GFb.

SGLT 1 and 2 IC_{50} determination

Unfortunately, glycogen phosphorylase inhibitors have not been developed into a drug for clinical use. However, based on Sebastian Vidal's summary article, the general structure of the SGLT2 inhibitors already on the market includes a glycopyranose ring with a benzene ring attached by a C-glycoside bond, and an aromatic group attached to the benzene ring by a methylene bridge. My colleagues have synthesized a number of glucose analogue inhibitors with a similar structure to commercially available SGLT2 inhibitors, but instead of the first aromatic ring, the molecule contains a heteroatomic ring, pyrimidine, oxadiazole, and triazole. Thus, I set out to investigate molecules containing a heteroaromatic group instead of a benzene ring as potential SGLT inhibitors.

Table 2: Compounds tested for SGLT1 and 2 inhibition

Code	Structure	Code	Structure
17		**18**	
19a		**19b**	

Code	Structure	Code	Structure
19c		**20a**	
20b		**20c**	
21a		**21b**	
21c		**21d**	
22		**23a**	
23b		**23c**	
24			
25a		**25b**	
26a		**26b**	

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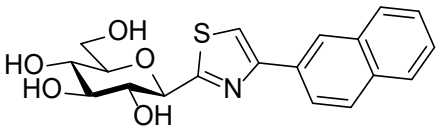
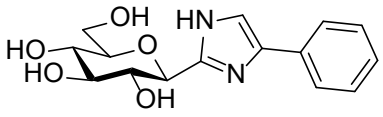
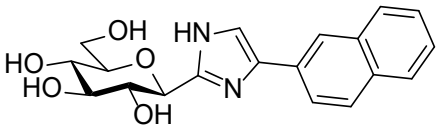
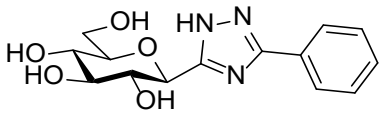
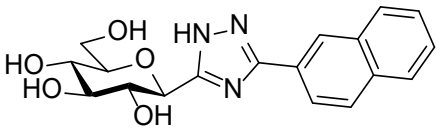
27a		**27b**	
28a		**28b**	
29a		**29b**	

To measure the SGLT inhibitory effects of the compounds, we generated two new stable cell lines using lentiviral vectors that express high levels of SGLT1 and SGLT2 proteins. Transfection results were verified by western blotting. The results show a significant increase in the amount of the protein of interest.

In addition, the glucose uptake of the cells after transfection was also examined. The cells were treated with 2-NBDG. The amount of glucose uptake nearly doubled in both cell lines. The cell lines were validated with dapagliflozin and the effect of the inhibitor phlorizin, SGLT 1 and 2 was further investigated. The glucose uptake of the treated group was significantly reduced compared to that of the untreated group.

The glucose derivative inhibitors used in our experiments were provided by László Somsák and his research group. We tested the cytotoxicity of the molecules, which had inhibitory activity. No cytotoxicity was observed for any of the compounds.

The tested compounds were divided into 2 groups, one group consisting of newly synthesized compounds and the other group of compounds previously tested for glycogen phosphorylase inhibition. Among the members of the first group, compounds capable of inhibiting in the micromolar range were identified (19a, 20a, 22 and 23c). These compounds showed a weaker inhibitory activity than the known C-glucosyl-arene type inhibitor, dapagliflozin (18), but their activity exceeded that of phlorizin (17). In addition, previous observations that substitution of the proximal aromatic ring of C-glucosyl(het)arene-type SGLT2 inhibitors with heterocycles reduces the inhibitory efficacy, the extent of which is strongly dependent on the heteroaromatic ring structure, were confirmed.

In the benzyl substituted oxadiazole compounds, the presence of the oxadiazole ring did not significantly affect the inhibitory effects (19a and 20a). Replacement of the oxygen atom of the heterocyclic oxadiazole with an H-bonding NH group (resulting in a corresponding 1,2,4-triazole) led to a 20-30-fold weakening of the inhibition (23a vs. 19a and 20a). Insertion of a phenyl ring on the N1 atom of 1,2,4-triazole significantly increased the efficiency (23c vs. 23a), while insertion of a hydroxyethyl group in the same position caused a complete loss of inhibition (23a vs. 23b). Compared with 23a (1,2,4-triazole), which also contains an NH group, 22 (imidazole) was found to be ~20-fold more effective.

The role of the NH group seems to be controversial, as compound 22 is a stronger inhibitor, while 23a is weaker. It is hypothesised that this may be the result of three possible tautomeric forms of 23a, 1,2,4-triazole, only one of which may bind to the protein, while in the case of 22, imidazole, the formally existing tautomers are essentially identical due to a possible protonation in the biological medium.

Furthermore, the inhibitory effect is highly dependent on the substitution of the distal aromatic ring of the inhibitor molecule. In the case of 1,2,4- and 1,3,4-oxadiazoles, only unsubstituted derivatives (19a and 20a) are able to inhibit while the introduction of a methoxy or chloro group into the para position of the benzene ring, for example in the case of 19b, 20b and 19c and 20c, led to a complete loss of inhibition.

In the pyrimidine series, in addition to the benzyl derivative (21a), the 4-methoxybenzyl derivative (21b) also exhibited an inhibitory effect in the micromolar range. However, compound 21c, which is a 4-chlorobenzyl derivative, had no inhibitory activity. Substitution of the distal benzene ring with a 2-naphthyl group also resulted in a molecule (21d) with no inhibitory activity.

The compounds discussed so far (17-24) do not possess significant rabbit skeletal muscle GFb enzyme inhibitory activity, but compounds 25-29 are a group of GF inhibitors previously investigated by our working group. The compounds were selected from non-inhibitory to GF inhibitors capable of inhibiting in the low nanomolar range. The molecules of compounds 25-29 contained 2-naphthyl (25b - 29b) and phenyl groups (25a - 29a) as distal aromatic groups. From the results of the measurements, it can be concluded that the 27 thiazoles were ineffective against both transporters tested. The other compounds had variable inhibitory activity, indicating the importance of the heterocyclic group.

Most of the compounds tested inhibited SGLT2 more than SGLT1 in the selectivity range 2-27. There were two exceptions, compounds 26b and 29b, both 2-naphthyl substituted,

inhibited SGLT1 more. In two comparisons, 25a - 25b and 28a - 28b, the 2-naphthyl derivatives inhibited SGLT2 ~7 and 26 times more strongly than SGLT1.

Both studies show that, despite the growing importance of modern computer-based molecular design, in vitro and in vivo experiments cannot be avoided, even in the most precise settings. In in vitro and in vivo systems, the structure of molecules and macromolecules is strongly influenced by their environment, such complex effects cannot be fully modelled, and therefore simplified systems are often used. Thus, both studies demonstrate that computational molecular design can accelerate the identification of potential compounds, but alone are not enough to develop new drug molecules, in vitro experiments cannot be avoided.

Summary

The liver plays an important role in maintaining proper blood glucose levels by regulating blood glucose levels through gluconeogenesis and glycogen synthesis/degradation. A key enzyme in glycogen degradation is glycogen phosphorylase, which catalyses the first step of degradation. The enzyme has 5 ligand binding sites through which specifically designed molecules can inhibit the activity of the enzyme.

Another important organ for maintaining adequate blood glucose levels is the kidney. In the proximal tubule of the kidney, there are two types of sodium-dependent glucose cotransporters (SGLT1 and 2) responsible for glucose reabsorption. Considering the physiological functions of SGLT1 and SGLT2, they have become reasonable targets for drug discovery in the 2000s and several clinical drugs are currently in use. These SGLT2 inhibitors inhibit glucose reabsorption, thereby reducing blood glucose levels and increasing urinary excretion of glucose.

Rapid advances in science over the last decade have made it possible for researchers to design inhibitors using computers without in vitro or in vivo experiments. Joseph M. Hayes and colleagues performed such computer screening to identify potential glycogen phosphorylase inhibitor molecules, which inhibit the active site of the enzyme. From the compounds screened, the 7 most promising molecules were selected and synthesized by Dr. László Somsák and his team. One of our aims of the present work was to perform kinetic measurements of the selected compounds and compare the computer-generated results with those obtained in in vitro experiments.

In our studies we found that compounds with measured K_i competitively inhibit the active site of the enzyme. Compound 5c, which was predicted by simulation to be the best inhibitor, had the lowest measured K_i (1.97 μM), but fell short of the predictions of the computer calculations. The potency of compounds 13b (K_i = 4.58 μM) and 12b (K_i = 26.2 μM) corresponded to the calculations but 13a (K_i = 68.6 μM) and 13c (K_i = 71.1 μM) fell short of the predicted value. Compounds 12a (K_i = 326 μM) and 12c (K_i = 540 μM) proved to be much weaker inhibitors than expected based on the calculations.

In the second part of my work, I investigated the SGLT1 and 2 inhibitory activity of glucose analogue inhibitor compounds. In the selection of the compounds tested, we took into account Sebastian Vidal's summary article, which states that glycogen phosphorylase inhibitors similar to the structure of SGLT2 inhibitors used in clinical practice may also be able to inhibit the SGLT2 protein. The general structure of the SGLT2 inhibitors already on the market

includes a glycopyranose ring with a benzene ring linked by a C-glycoside bond, and an aromatic group linked to the benzene ring by a methylene bridge. My colleagues have synthesized a number of glucose analogue inhibitors with similar structures to commercially available SGLT2 inhibitors, but instead of the first aromatic ring, the molecules contain a heteroatomic ring, pyrimidine, oxadiazole, imidazole, thiazole, or triazole. To perform this study, stable cell lines, which overexpress SGLT1 and SGLT2 protein, were generated by lentiviral transfection. The success of the transfection was demonstrated by Western blot and 2-NBDG uptake. Furthermore, the cell lines were validated by dapagliflozin. The tested compounds showed no cytotoxicity. The tested compounds were divided into 2 groups, one group consisting of newly synthesized compounds and the other group of compounds previously tested for glycogen phosphorylase inhibition.

Among the members of the first group, we identified some new SGLT inhibitors with IC_{50} values in the micromolar range, but lagging behind the inhibitory activity of known C-glucosyl-arene type inhibitors. Substitution of the proximal aromatic group, oxadiazole, for triazole led to a 20-30-fold attenuation of inhibition (23a vs. 19a and 20a). Insertion of a phenyl ring on the N1 atom of 1,2,4-triazole resulted in a significant efficiency improvement (23c vs. 23a), while switching a hydroxyethyl group to the same position caused a complete loss of inhibition (23a vs. 23b). Compared with 23a (1,2,4-triazole), which also contains an NH group, 22 (imidazole) was found to be ~20-fold more effective.

In the second group, compounds containing thiazole showed no inhibitory effects. Most of the compounds inhibited SGLT2 more than SGLT1, except compounds 26b and 29b, which inhibited SGLT1 more. Furthermore, in the comparisons 25a - 25b and 28a - 28b, the 2-naphthyl derivatives, inhibited SGLT2 more strongly than SGLT1. Compound 28b, with an SGLT2 IC_{50} of 3.5 M, was found to be the best inhibitor, and considering the nanomolar inhibition of GF, compound 28b is a multi-target compound that could be a starting point for further antidiabetic studies.

APPENDIX

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Subject: PhD Publication List

Candidate: Ádám Sipos
Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. **Sipos, Á.**, Szennyes, E., Hajnal, É., Kun, S., Szabó, E. K., Uray, K., Somsák, L., Docsa, T., Bokor, É.: Dual-Target Compounds against Type 2 Diabetes Mellitus: proof of Concept for Sodium Dependent Glucose Transporter (SGLT) and Glycogen Phosphorylase (GP) Inhibitors. *Pharmaceuticals. 14* (4), 1-27, 2021.
   DOI: http://dx.doi.org/10.3390/ph14040364
   IF: 5.863 (2020)
2. Barr, D., Szennyes, E., Bokor, É., Al-Oanzi, Z. H., Moffatt, C., Kun, S., Docsa, T., **Sipos, Á.**, Davies, M. P., Mathomes, R. T., Snape, T. J., Agius, L., Somsák, L., Hayes, J. M.: Identification of C-[béta]-d-Glucopyranosyl Azole-Type Inhibitors of Glycogen Phosphorylase That Reduce Glycogenolysis in Hepatocytes: in Silico Design, Synthesis, in Vitro Kinetics, and ex Vivo Studies.
   ACS Chem. Biol. 14 (7), 1460-1470, 2019.
   DOI: http://dx.doi.org/10.1021/acschembio.9b00172
   IF: 4.434

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List of other publications

3. Docsa, T., Bhattarai, D., **Sipos, Á.**, Wade, C. E., Cox, C. S., Uray, K.: CXCL1 is upregulated during the development of ileus resulting in decreased intestinal contractile activity.
Neurogastroenterol. Motil. 32 (3), 1-12, 2020.
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J. Agric. Food Chem. 67 (24), 6884-6891, 2019.
DOI: http://dx.doi.org/10.1021/acs.jafc.9b00443
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5. Szabó, E. K., Kyriakis, E., Psarra, A. M. G., Karra, A. G., **Sipos, Á.**, Docsa, T., Stravodimos, G. A., Katsidou, E., Skamnaki, V. T., Liggri, P. G. V., Zographos, S. E., Mándi, A., Király, S. B., Kurtán, T., Leonidas, D. D., Somsák, L.: Glucopyranosylidene-spiro-imidazolinones, a New Ring System: Synthesis and Evaluation as Glycogen Phosphorylase Inhibitors by Enzyme Kinetics and X-ray Crystallography.
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6. Szennyes, E., Bokor, É., Docsa, T., **Sipos, Á.**, Somsák, L.: Synthesis of C-β-D-glucopyranosyl derivatives of some fused azoles for the inhibition of glycogen phosphorylase.
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7. Kun, S., Begum, J., Kyriakis, E., Stamati, E. C. V., Barkas, T. A., Szennyes, E., Bokor, É., Szabó, E. K., Stravodimos, G. A., **Sipos, Á.**, Docsa, T., Gergely, P., Moffatt, C., Patraskaki, M. S., Kokolaki, M. C., Gkerdi, A., Skamnaki, V. T., Leonidas, D. D., Somsák, L., Hayes, J. M.: A multidisciplinary study of 3-(β-D-glucopyranosyl)-5-substituted-1,2,4-triazole derivatives as glycogen phosphorylase inhibitors: computation, synthesis, crystallography and kinetics reveal new potent inhibitors.
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8. Kun, S., Bokor, É., **Sipos, Á.**, Docsa, T., Somsák, L.: Synthesis of New C- and N-β-d-Glucopyranosyl Derivatives of Imidazole, 1,2,3-Triazole and Tetrazole, and Their Evaluation as Inhibitors of Glycogen Phosphorylase.
Molecules. 23 (3), 1-17, 2018.
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New J. Chem. 42 (21), 17439-17446, 2018.
DOI: http://dx.doi.org/10.1039/C8NJ04035D
IF: 3.069

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