SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

STUDY OF ANTIHYPERGLYCAEMIC AGENTS ON THE INHIBITION OF GLYCOGEN PHOSPHORYLASE

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1. INTRODUCTION

Type 2 diabetes (non-insulin-dependent diabetes mellitus) is a prevalent disease, afflicting about 140 million people world-wide. The disease is characterized by chronic hyperglycaemia and insulin resistance. Type 2 diabetes can be treated with lifestyle changes (diet and exercise, for example), with drugs that promote insulin release and with insulin. Tight control of blood glucose level prevents and delays the onset of diabetic complications but such a control is rarely achieved with oral antidiabetic agents. Safer and more effective therapy is urgently needed. Glycogen phosphorylase (GP; EC 2.4.1.1) appears as a potential target for such efforts because of its essential roles in glycogen metabolism and control of hepatic glucose output.

The liver is the predominant source of blood glucose in fasting type 2 diabetes. Hepatic glucose output is regulated by a complex system of enzymes. The main regulatory enzyme of this system is glycogen phosphorylase and only the phosphorylated enzyme (GP*a*) has significant activity. GP*a* releases glucose-1-phosphate from glycogen as shown in Fig. 1, suggesting an important role for glycogenolysis in hepatic glucose production. Gluconeogenesis from lactate and other precursor molecules can also contribute to the elevated blood glucose levels, however, it was clearly demonstrated that glucose arising from gluconeogenesis has cycled through glycogen. Therefore the inhibition of hepatic GP could suppress glucose production arising from both glycogenolysis and gluconeogenesis.

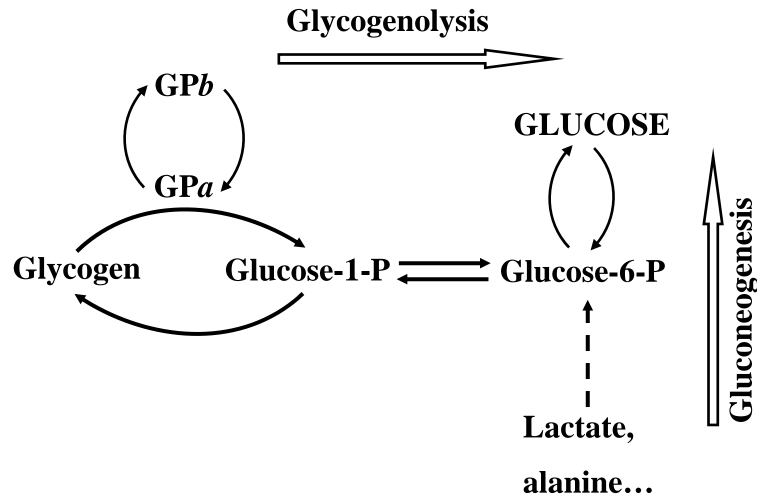


Figure 1. Some details of hepatic glycogenolysis and gluconeogenesis. GP*a* and GP*b* represent the phosphorylated, active form and nonphosphorylated, inactive form of the enzyme, respectively.

The enzyme system of glycogen metabolism is under hormonal, neuronal, and metabolic control. Positive effectors promote degradation of glycogen (e.g. glucagon, AMP), while negative effectors (e.g. glucose, glucose-6-phosphate, ATP) retard degradation. The catalytic activity of GP can be inhibited by glucose and other glucose-based compounds, which bind at the active site, and by caffeine and analogous heteroaromatic derivatives which bind at the purine inhibitory site, also called the I-site.

Type 2 diabetes (non-insulin-dependent diabetes mellitus) is a major public health problem with severe complications, such as cardiovascular disease, neuropathy, retinopathy and nephropathy. Type 2 diabetes comprises approximately 90-95% of all diabetes cases. Diabetes affects approximately 6-20% of the adult population in Western industrialized societies and is a major public health threat, reaching epidemic rates. The disease is polygenic and is characterized by hyperglycaemia, defects in insulin secretion and insulin resistance, in which the tissues fail to respond to this hormone. Additionally, the rate of endogenous glucose production is elevated in type 2 diabetes.

The liver accounts for 90% of the body's endogenous glucose production and is tightly controlled by insulin and glucagon. In type 2 diabetes, hepatic insulin resistance with elevated amounts of glucagon results in high levels of glucose,

which in turn contributes to the observed hyperglycaemia. Hepatic glucose is produced via two pathways: glycogenolysis (breakdown of glycogen) and gluconeogenesis (*de novo* synthesis of glucose). Studies estimate the glycogenolytic contribution of total hepatic glucose production to be up to 75% in both healthy controls and patients with type 2 diabetes. Furthermore, a substantial portion of glucose formed by gluconeogenesis is cycled through the glycogen pool prior to efflux from the liver cells. The inhibition of either glycogenolysis or gluconeogenesis appears to be an attractive approach to developing antihyperglycaemic molecules.

Glycogen phosphorylase (GP), which catalyzes the first reaction in the degradation of glycogen, is considered a potential target for type 2 diabetes, generating selective ligands of GP that modulate the activity of this protein.

The accumulation and breakdown of glycogen are regulated by the reciprocal activities of glycogen synthase (GS) and GP. Only the phosphorylated GP*a* has significant activity, suggesting an important role for glycogenolysis in hepatic glucose production. Therefore, the inhibition of hepatic GP might suppress glucose production arising from glycogenolysis and gluconeogenesis. It is known that the activity of liver GS is also controlled by reversible phosphorylation of multiple serine residues. The dephosphorylated form of GS is the catalytically active (GS*a*) form. Phosphorylation is associated with the inactivation of GS: conversion to the *b* form. The dephosphorylation of GP and GS is interrelated and is catalyzed by the glycogen-associated protein phosphatase-1. GP*a*, but not GP*b*, is a potent inhibitor of the phosphatase action on GS, and it is only when GP*a* has been dephosphorylated that the phosphatase is free to activate GS, the rate limiting enzyme of glycogen synthesis.

Rodents are commonly used for diabetic studies. Streptozotocin, a monofunctional nitrosourea derivative, is one of the most frequently applied substances to induce diabetes in experimental animals. Genetic models of insulin resistance and hyperglycaemia are also available, including Zucker diabetic fatty (ZDF) rats. These rats have a mutation in the leptin receptor and a defect in the pancreatic β cells that affect insulin production.

There are several binding sites in GP that lend themselves to targeting by various effectors. Many glucose derivatives were shown to bind to the catalytic

centre of GP, and among them glucopyranosylidene-spiro-hydantoin with a *K*i of 3-4 μM was one of the most efficient inhibitors. More recent efforts resulted in the synthesis of a thio-analogue of the molecule with a very similar *K*i value. In the present study, we report the effect of D-glucopyranosylidene-spiro-thiohydantoin (TH) on glycogen metabolism in the liver tissues of streptozotocin-induced and obese diabetic rats.

2. OBJECTIVES

There are several binding sites in GP lending themselves to be targeted by various effectors. Many glucose derivatives were shown to inhibit the catalytic activity of GP, and among them glucopyranosylidene-spiro-thiohydantoin (TH) with a K_i of 4-6 μM was one of the most efficient inhibitor. Several further molecules have been synthesized and the study of their inhibitory potential as well as the biological assessment of inhibitors *in vivo* using various animal models (spontaneous diabetic rats, streptozotocin-treated rats) are also in the focus of the present study.

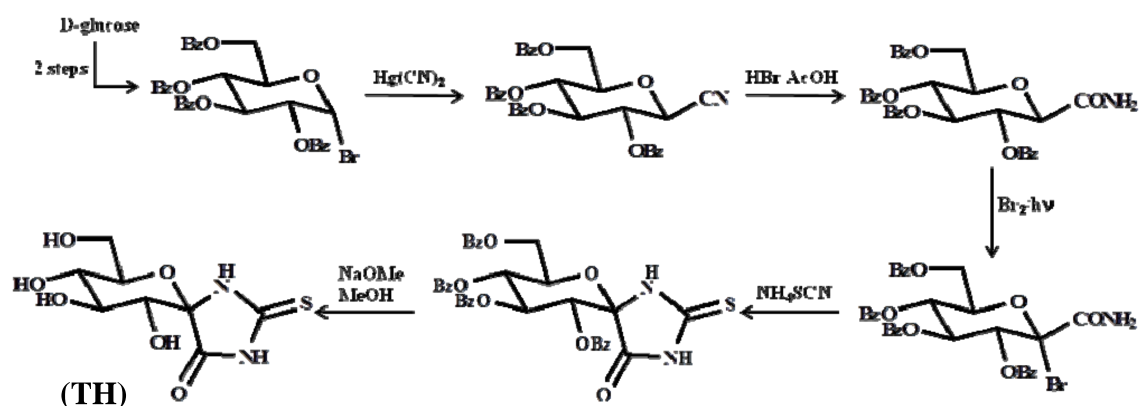
Major aims of the present study were as follows.

- Investigation of glucose analogue molecules on the catalytic activity of muscle and liver GP isoforms.
- Kinetic analysis of the inhibition: determination of K_i and/or IC_{50}.
- X-ray data collection, crystal structure determination using muscle GPb-ligand complexes (collaborative study).
- Structure and inhibitory potential analysis for the design of further glucose analogues.
- Study of glycogen metabolism in the liver of Wistar rats (assay of blood glucose and liver glycogen; activity of GP and glycogen synthase (GS), dephosphorylation of these enzymes in the presence of inhibitor molecules).
- Investigation of the effect of TH in various diabetic animal models (streptozotocin-induced and spontaneous diabetic Zucker rats) to establish the antihyperglycaemic effect. Investigation of TH on the plasma insulin level and insulin sensitivity of diabetic animals.

3. MATERIALS AND METHODS

Synthesis of glucopyranosylidene-spiro-thiohydantoin

The synthesis of glucopyranosylidene-spiro-hiohydantoin based on six step, simple, highly chemo-, region-, and stereoselective procedure starting from D-glucose by Prof. Laszló Somsak and his group (University of Debrecen, Organic Chemistry Department).



Synthesis of glucopyranosylidene-spiro-thiohydantoin

Determination of inhibitory constants (K_i and IC_{50})

Enzyme activity was assayed into the physiological direction of glycogen breakdown. Kinetic data were collected using muscle or liver glycogen phosphorylase isoforms in the phosphorylated (activated: GP*a*) and dephopshorylated (GP*b*) isoforms. Kinetic data for the inhibition of phosphorylases by glucose analogues were obtained at varying concentrations of D-glucose-1-phosphate and constant concentration of glycogen. Enzymatic activities were presented in the form of double-reciprocal plot (Lineweaver-Burk). The plots were analysed by a non-linear data analysis program. The inhibitor constants (Ki) were determined by secondary plots, replotting the slopes from the Lineweaver-Burk plot against the effector concentrations. The means of standard errors for all calculated kinetic parameters averaged to less than 10%.

X-ray crystallography

Native T-state rabbit muscle GP*b* was used to investigate the enzyme-ligand interaction by X-ray crystallography. For the preparation of the GPb-inhibitor complex the enzyme was soaked in 100 μM inhibitor containing buffer for few hours. X-ray data were collected in Hamburg using X-31 beam (λ= 1.05-1.97 Å). Diffraction studies were made by our collaborating partner (The National Hellenic Research Center, Athens, Greece). The collected data were analyzed by SYBYL programme (Tripos Associates Inc., St Louis, MO, USA).

Streptozotocin treatment and body weight measurement

Male Wistar rats weighing ~250 g were obtained from Charles River Magyarország Kft. (Budapest, Hungary). Diabetes was induced by a simple intravenous injection of a freshly prepared solution of streptozotocin (60 mg/kg body weight) dissolved in 50 mM sodium-citrate (pH 4.5) plus 150 mM NaCl. Control rats were injected with citrate buffer alone. Diabetes was assessed periodically by a test for glucosuria (Macherey-Nagel, Düren, Germany). One week after diabetes induction, the animals were determined to be diabetic if urine glucose levels exceeded 3 mg/ml. The diabetic animals were randomly divided into two groups: control diabetic and TH-treated diabetic. The control and the streptozotocin-injected group were subcutaneously administered 0.9% NaCl (2 ml/kg body weight) and the TH-treated streptozotocin-injected group was subcutaneously administered 50 μM TH solution (100 μM/kg body weight). TH was freshly dissolved in 0.9% NaCl solution before the treatment. Treatments were performed 10 times between 8 and 9 a.m. every second day. Rats were anesthetized on the second day after the 10th treatment, the livers were removed by median laparotomy, and the wet liver weight was measured. ZDF/Gmi-fa/fa (ZDF) inbred obese male rats from Genetic Models, Inc. (Indianapolis, IN, USA) were ordered through Charles River Hungary Kft.

All animals were cared for observing standard procedures with permission of the Laboratory Animals Protection Committee (29/2007/DEMÁB).

Gel filtered liver extract

The liver samples were homogenized in a Potter-Elvehjem tube in two-volume ice-cold buffer consisting of 250 mM sucrose and 40 mM Tris-HCl (pH 7.4), and centrifuged at 10,000 x g for 10 min. The resulting post-mitochondrial supernatant was filtered through a Sephadex G-25 column (5x1.5 cm) to remove endogenous effectors, equilibrated in 40 mM Tris-HCl, 1 mM EDTA and 10 mM mercapto-ethanol (pH 7.4). The filtrates were supplemented with 5 mM $(NH_4)_2SO_4$ and 1 mM magnesium acetate in the presence of various effectors and incubated at 30˚C. Samples were withdrawn at the indicated times for the assay of GP or GS activites.

Hyperinsulinaemic and euglycaemic glucose clamp

The animals were prepared as described above. Fasting blood sample for blood glucose and plasma insulin was obtained before the hyperinsulinaemic euglycaemic glucose clamp has commenced. Beside continuous insulin infusion (Humulin R®, Eli Lilly, Indianapolis, IN; USA) at constant rate of 3 mU/kg/min for 120 min, the 20% glucose infusion was adjusted to maintain the euglycaemic (5.5±0.5 mol/l) state. Blood samples (0.1 ml) were obtained from the carotid artery for determination of blood glucose concentration before and at 5 min intervals during the first 80 min of the clamp and at 10 min intervals during the last 40 min of the clamp. Immediately before the insulin infusion was started a carotid arterial blood sample (0.5 ml in 20 µl EDTA and 10 µl Trasylol; Bayer, Leverkusen, Germany) was collected, centrifuged and the plasma aliquoted, frozen and stored at -70 ºC for subsequent determinations of plasma insulin levels by radioimmunoassay. Steady state insulin levels were also determined same way as described above at 100 and 120 min, respectively. The glucose infusion rate needed to maintain this euglycaemic blood glucose target level was used to characterize peripheral insulin sensitivity.

4. RESULTS

Structure – inhibitory efficiency of glucose analogue compounds

TH, one of the most effective glucose analogue inhibitor for GPb, has a K_i = 5.2 μM, more than 500 times lower than the corresponding K_i (1.7 mM) for glucose. TH binds at the catalytic site with the spiro-thiohydantoin group filling the empty space at the β configuration adjacent to C1 of glucopyranose as defined previously. Assays with muscle and liver GP*b* and GP*a* enzyme render TH to be equipotent inhibitors. The two subunits of the functionally active dimer are related by a crystallographic 2-fold symmetry axis. The structure of the GP*b*-TH complex has been refined at 2.26 Å to a crystallographic R value 0.193 (R_{free}=0.211). TH binds at the catalytic site which is buried some 15 Å from the surface of the enzyme at the bottom of a long channel.

TH, on binding to GP*b*, makes a total of 15 hydrogen bonds - an additional rather long hydrogen bond (3.4 Å) is made between the TH Asn284. Furthermore, van der Waals interactions (3 nonpolar/nonpolar, 13 polar/polar, and 45 polar/nonpolar) can be also depicted. The structural results show that sulphur can be accommodated in this position with essentially no disturbance of the structure. There are no changes at the allosteric site, at the inhibitor site or at the tower/tower subunit interface.

N-benzoyl-N'-β-D-glucopyranosyl-urea (Bzurea) binds at the catalytic site with the urea and benzoyl moieties filling the empty space of the so called β-pocket, a side channel from the catalytic site that leads toward residue His341, but with no access to the bulk solvent. There are in total 21 hydrogen bonds and 96 van der Waals interactions (7 nonpolar/nonpolar, 16 polar/polar, and 73 polar/nonpolar) in the GPb-Bzurea complex. There is no hydrogen bond between the amide of Bzurea and His377. N-atoms are hydrogen-bonded to Asp339 through two water molecules, which is also hydrogen-bonded to His341 of the β-pocket. O-atom of the carbonyl group contacts Leu136 and Gly135 and Arg569 through water molecule, and there are also water-mediated contacts to Gly134 and carboxyl and Asp283, which undergoes conformational change. There are negligible changes in the overall conformation on binding Bzurea to GP*b*, but there is a dramatic shift in

the 280s loop with concomitant changes in the adjacent imidazole of His571, and some differences in the water structure at the catalytic site.

Bzurea, on binding to GPb, also occupies the new allosteric inhibitor site, the site that is almost buried in the central cavity of the enzyme. The new allosteric site, identified recently as a target for drug interactions, has been shown to bind a number of indole-2-carboxamide inhibitors. The conformation of the bound Bzurea is not identical with that described above for the catalytic site. In addition, the phenyl ring is coplanar to the urea moiety, whereas, at the catalytic site, the plane of the phenyl ring is inclined 30° to the plane of the urea moiety. Bzurea on binding at the new allosteric inhibitor site of GPb makes a total of nine hydrogen bonds and exploits 80 van der Waals interactions, 28 of which are interactions between nonpolar groups; in total, there are 21 contacts with the symmetry related subunit. The benzoyl moiety exploits 43 van der Waals contacts (25 nonpolar/nonpolar), which are dominated by the substantial contacts.

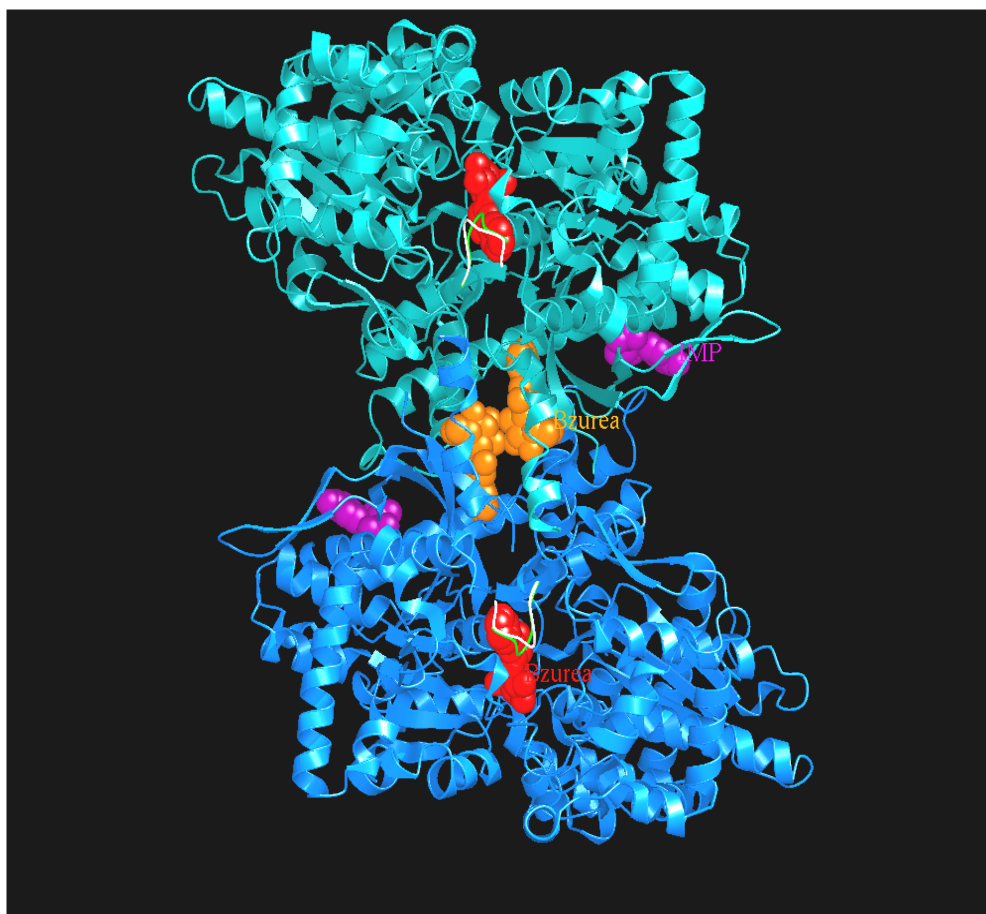


Figure 2. Binding of benzoyl-urea to the dimeric GPb enzyme

Effect of TH on hepatic glycogen metabolism

It is known that glucose and glucose analogues cause a sequential inactivation (dephosphorylation) of liver GP*a* and activation (dephosphorylation) of GS*b*. The coordinated regulation of GP and GS was tested in gel-filtered rat liver extract in the presence of 20 mM glucose or 50 μM TH. Both ligands enhanced the dephosphorylation (inactivation) of hepatic GP*a*, however, TH applied at a much lower concentration appeared to be more effective. We also investigated the effect of either glucose or TH on the dephosphorylation (activation) of GS*b* in gel-filtered rat liver extract. TH significantly increased the amount of the active form of hepatic GS by decreasing the latency in the dephosphorylation of GS*b*.

Streptozotocin is widely utilized to induce type 1 diabetes in rats with concomitant insulin deficiency, which is considered to be one of the animal models of human diabetes mellitus. One week after the injection of streptozotocin the average blood glucose level was about 30 mM in the diabetic group and remained at this concentration during the experimental period of 4 weeks. Hepatic glycogen content dramatically decreased in diabetic animals from 24 mg/g to 2 mg/g. Diabetic animals treated with 100 μM/kg body weight of TH exhibited significantly lower serum glucose concentration, and the hepatic glycogen content was also restored.

The co-ordinated control of GP and GS in gel-filtered liver extracts in the various experimental groups was also investigated. The diabetic group showed higher GP*a* activity compared to the control animals, while GS activity was markedly suppressed in the streptozotocin-treated rats. TH-treated diabetic rats had the lowest GP*a* activity, however, GS*a* activity was rather high, supporting the fact that glycogen concentration was elevated in the liver of this group.

The ZDF rat develops overt diabetes with severe hyperglycaemia, polyurea and polydipsia. Therefore, it is a good type 2 diabetic model for testing small molecule antidiabetic compounds. The co-ordinated control of GP and GS was observed in the gel-filtered extracts of spontaneously diabetic rats. When the GP*a* level was lowered, GS*b* was sequentially dephosphorylated, and through this the activation of glycogen synthesis was initiated. Intravenous administration of TH significantly decreased the GP*a* level and the activation of GS was started without any delay.

5. SUMMARY

The major role of hepatic glycogen is to supply glucose to the circulation maintaining the normal blood glucose level. In muscle and liver, the accumulation and breakdown of glycogen are regulated by the reciprocal activities of glycogen phosphorylase and glycogen synthase. Glycogen phosphorylase catalyses the key step of glycogen degradation and its activity can be inhibited by glucose and its analogues. Obviously, any readily accessible inhibitor of glycogen phosphorylase can be used as a potential therapy of non-insulin-dependent or type 2 diabetes. Hepatic glycogen phosphorylase has been identified as a new target for drug design that control blood glucose concentration.

One of the aims of our present study was studying the newly synthesized glucose analogue inhibitors by assaying the inhibitory potential with purified rabbit muscle or rat liver glycogen phosphorylases. Our investigations showed that spiro-thiohydantoin derivatives are very potent inhibitors with K_i values in the low micromolar range. Furthermore, substituted urea compounds have been identified as good inhibitors also binding to the active centre and the recently discovered new allosteric binding site of glycogen phosphorylase as revealed by crystallographic studies. By the assay of several glucose derivatives as possible inhibitors of glycogen phosphorylase structure-activity relationships were analyzed.

Glucopyranosylidene-spiro-thiohydantoin (TH) was found as one of the most potent glucose derivate which inhibits the catalytic activity of both muscle and liver glycogen phosphorylase. We have demonstrated the co-ordinated regulation of glycogen phosphorylase and synthase by 50 μM TH in liver extracts of Wistar rats resulting in the activation of synthase by shortening the latency compared with control animals. TH was also effective in lowering blood glucose level and restoring hepatic glycogen content in streptozotocin-induced diabetic rats. Furthermore, intravenous administration of TH to Zucker diabetic fatty rats significantly decreased the hepatic glycogen phosphorylase *a* level and the activation of synthase started without any delay.

TH was also tested on the insulin sensitivity and blood glucose level of control and streptozotocin-treated rats. TH increased the insulin sensitivity and also contributed to the partial restoration of blood glucose level of treated animals proving its efficiency as a possible antihyperglycaemic agent.

6. APPENDIX

DEBRECENI EGYETEM EGYETEMI ÉS NEMZETI KÖNYVTÁR
KENÉZY ÉLETTUDOMÁNYI KÖNYVTÁRA

Iktatószám: DEENKÉTK /306/2011.
Tételszám:
Tárgy: Ph.D. publikációs lista

Candidate: Tibor Docsa

Neptun ID: FI17ZV

Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. **Docsa, T.**, Czifrák, K., Hüse, C., Somsák, L., Gergely, P.: Effect of glucopyranosylidene-spiro-thiohydantoin on glycogen metabolism in liver tissues of streptozotocin-induced and obese diabetic rats.
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