Short thesis for the degree of doctor of philosophy (PhD)

Difficulties and their solutions associated with the *in vitro* assays of glycoenzyme-inhibitors applicable for treating type 2 diabetes mellitus

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Abbreviations

ABI	aggregation-based inhibition
AMP	adenosine monophosphate
API	aggregation-prone inhibitor
CNP-G7	2-chloro-4-nitrophenyl-β-D-maltoheptaoside
DLS	dynamic light scattering
EC_{50}	half-maximal effective concentration
$GalG_2CNP$	2-chloro-4-nitrophenyl O-β-D-galactopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-α-D-glucopyranoside
GP	glycogen phosphorylase
GTH	glucopyranosylidene-spiro-thiohydantoin
G-1-P	glucose-1-phosphate
ITC	isothermal titration calorimetry
k_{cat}	catalytic rate constant (turnover number)
K_i	inhibitory constant
K_i^*	apparent inhibitory constant
K_M	Michaelis constant
P_i	inorganic phosphate
PPA	porcine pancreatic α-amylase
v_{max}	maximal reaction rate

I. Introduction and objectives

Despite the current availability of many glycoenzyme-inhibitors as antidiabetic agents, new inhibitors are still being intensively searched for. The primary enzyme targets for the treatment of type 2 diabetes mellitus are glycogen phosphorylase (GP) and α-amylase. In my doctoral dissertation, I dealt with the two most problematic parts of the *in vitro* studies of glycoenzyme-inhibitors, namely with the application of an adequate method for measuring enzymatic activity and with the deduction of correct conclusions from experimental results.

Working out a new activity measurement method often represents a considerably complex process. In the case of GP, for instance, we need to take into account that the enzyme is present in an inactive form (GPb), therefore its activation needs to be provided. Since GP is an enzyme with two substrates, we also need to ensure that the reaction will be of pseudo-zero order for one of the substrates. In addition, this enzyme is capable of catalysing both the degradation and synthesis of glycogen, consequently, we need to find a technique that makes it possible to investigate both directions. I set myself the objective of working out a new activity measurement method that allows following both phosphorolytic and synthetic processes under identical circumstances. Using the same reaction conditions, the inhibitory parameters of a compound can be compared, allowing us to determine whether the compounds examined in the non-physiological direction are also effective in the case of the pharmaceutically relevant direction.

However, the development of an adequate method for measuring enzyme activity is not enough in itself; careful consideration must be given to the selection of the inhibitors to be utilized for inhibition studies, and experimental results should also be evaluated taking into account a number of aspects. This will help avoid the other frequently emerging problem in drug design and development, namely that the inhibitors already synthesized are commonly found to be 'dead-ends'. For drug development, one of the most unfavourable phenomena is the non-specific, 'promiscuous' nature of drugs (significant inhibitory activity exerted on various unrelated enzymes). In most cases, promiscuity can be accounted for aggregate formation, which may enhance the risk of the evolution of side effects. Nevertheless, there are a number of publications in literature where more and more remarkable therapeutic effects of already known compounds have been reported without investigating the molecular mechanism underlying that promiscuous behaviour. Moreover, there are some publications describing the extremely high biological efficacy of newly synthesized inhibitors, in spite of the fact that the compounds have such a backbone, which has repeatedly formed the structural unit of other aggregation-prone inhibitors (API). For this reason, I aimed to study the aggregation tendency of potential glycoenzyme-inhibitors of synthetic origin using direct and indirect strategies. By applying thiazolidinone derivatives having a rhodanine backbone modified with various substituents, I intended to reveal whether the non-specific, promiscuous nature of an inhibitor could be inferred from the preliminary examination of core structure and whether the inhibitory mechanism of promiscuous compounds was indeed due to aggregation.

II. Applied equipment and methods

I applied isothermal titration calorimetry (ITC) to study the substrate specificity of rabbit muscle glycogen phosphorylase, to determine the half-maximal effective concentration of activator necessary to activate GPb having no catalytic activity, and to analyse the inhibitory effects of glucopyranosylidene-spiro-thiohydantoin (GTH) as well as caffeine. ITC experiments were carried out using a MicroCal ITC200 microcalorimeter (Malvern, Worcestershire, UK). *dQ/dt* values determined from raw calorimetric signals can be considered as the direct measure of initial reaction rate. Microcal Origin 7.0 graphing and data analysis software (Northampton, MA, USA) was used in each case to evaluate experimental ITC data. Single injection ITC measurements were performed, where the injector, the reference cell, and the sample cell contained the enzyme, the buffer, and the substrate in buffer (as well as the inhibitors during inhibition assays), respectively. When measuring the activity of GP with ITC, I used the same conditions in both directions of the catalysed reversible reaction, only changing the glucose-1-phosphate (G-1-P) and inorganic phosphate (P_i) concentrations of the applied buffer.

The optimization of the reaction conditions of the method suitable for measuring GPb activity and the preliminary investigation of the inhibitory effect of GTH as well as caffeine on the GPb enzyme were performed by HPLC technique. An Agilent 1260 Infinity II device (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a quaternary pump, a degasser, an autosampler, and a diode array detector was utilized for the experiments. Separation was performed with a Hypersil ODS column (20 cm x 4.6 mm, 120 Å, 5 μm, Agilent) using isocratic elution (acetonitrile: water=12:88 v/v%) and a flow rate of 1 ml/min. The detection of 2-chloro-4-nitrophenyl-β-D-maltoheptaoside (CNP-G7) selected as substrate as well as the longer or shorter chromogenic maltooligomer products occurred at 302 nm. For each measurement, 2.5 mM CNP-G7 and 2.5 mM-25 mM G-1-P or phosphate (Na_2HPO_4) substrates were freshly dissolved in 35 mM β-glycerophosphate buffer (pH 6.8) containing 2.6 mM EDTA and 1 mM AMP. The homogenized mixture of enzyme and substrate was incubated for 5 min at 37°C, and then 5 μL of sample was injected onto the column every 15 min (4 times in total). The evaluation of obtained chromatograms was accomplished by means of Agilent ChemStation software (Agilent Technologies Inc., Santa Clara, CA, USA).

The promiscuity as well as the aggregation-based inhibitory mechanism of acarbose, gallotannin, and the seven 2-thioxo-4-thiazolidinone derivatives were investigated by spectrophotometric method. A temperature-controlled Jasco V55 double beam UV-visible spectrophotometer (Jasco Corporation, Easton, MD, USA) was applied for my kinetic and inhibition studies. For these experiments, I selected porcine pancreatic α-amylase (PPA) as a model enzyme and 2-chloro-4-nitrophenyl O-β-D-galactopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-α-D-glucopyranoside ($GalG_2CNP$) as a substrate. I detected at 400 nm wavelength the increase in absorbance induced by the chromophore group (CNP) released as a result of hydrolysis catalysed by PPA. Using the Time course measurement function implemented in Jasco Spectra Manager™ software (Jasco Corporation, Easton, MD, USA), I

determined the slope of the initial (linear) section of the obtained curve, that is, the change in absorbance per unit of time (*dAbs/min*), which is proportional to the initial rate of the catalysed reaction. Percentile residual enzyme activities were plotted as a function of the applied inhibitor concentrations, and then a curve was fitted to the obtained points by non-linear regression using Microcal Origin 7.0 software.

In order to reveal the fulfilment of the criteria for APIs, I repeated the inhibition assays carried out under the original reaction conditions so that (1) the buffer contained 0.01% or 0.1% Triton X-100 non-ionic detergent, (2) the enzyme and inhibitors were incubated for 5, 10, and 30 min before initiating the enzyme reaction by the addition of the substrate, (3) the enzyme was utilized in multiple-fold concentrations, (4) the inhibitor solutions were centrifuged for 30 min before the spectrophotometric examination of the inhibitory activity of supernatant.

To directly identify aggregation-based inhibition (ABI), I investigated the particle size of inhibitors using dynamic light scattering (DLS). A Zetasizer NanoZS DLS device (Malvern Panalytical Ltd, Worcestershire, UK) was applied for these experiments. Due to the heterodispersity evoked by aggregation, I utilized the intensity-based particle size distribution. Beside this value, I also determined the polydispersity index and photon count rate for each sample. I performed two sets of measurements for each inhibitor. First, I examined the buffer solution of the given inhibitor then I aimed to detect the changes in the protein size under the influence of the inhibitor. Prior to each measurement, the samples as well as the buffer itself were filtered using a syringe filter with a pore diameter of 22 μm (Millex®-GP; Millipore™, Burlington, MA, USA) in order to avoid the possible interfering effects of contaminants. Malvern DTS 7.12 software (Malvern Panalytical Ltd, Worcestershire, UK) was applied for data collection and the evaluation of results.

III. New scientific results

1. I developed a new isothermal titration calorimetric method for the measurement of glycogen phosphorylase activity, which can be applied in both directions of the enzyme-catalysed reversible reaction without the derivatization of substrate and/or the use of auxiliary enzymes.

ITC device, which detects the amount of the heat absorbed or released in a process, proved to be suitable for measuring the activity of GP in both directions of the enzyme-catalysed reversible reaction. This method allowed utilizing the natural substrate of GP enzyme (glycogen) without derivatization. Traditional ITC techniques applied for enzyme kinetic assays have not been extensively used for reversible enzyme reactions since the products formed during the process can take part in the reverse reaction as substrates. In the past, only the activity of hydrolase enzymes was determined with ITC, where the reverse reaction is negligible due to the presence of the large amount of water.

1.1. Using ITC method, I determined the half-maximal effective concentration of activator necessary for the activation of glycogen phosphorylase b having no catalytic activity, in the directions of both phosphorylation and glycogen synthesis.

GPb, which does not have catalytic activity, can be activated in an allosteric manner with adenosine monophosphate (AMP). In order to determine the AMP concentration necessary for the effective action of the enzyme, I examined the activation effect of AMP, in the directions of both phosphorolysis and synthesis, with the use of CNP-G7 as a substrate. Considering the fact that P_i and G-1-P released during synthesis and breakdown, respectively can also be the substrates of the enzyme, I had to ensure that the reaction would be of pseudo-zero order related to these compounds. According to my measurements, the half-maximal effective concentration of activator (EC_{50}) was 50 μM for glycogen degradation and 26 μM for synthesis.

1.2. In order to characterize the substrate specificity of rabbit muscle glycogen phosphorylase, I determined the k_{cat}/K_M ratios representing the catalytic efficiency of the enzyme in the case of four substrates with different chain lengths.

As a first step in the development of the new ITC method, I investigated the substrate specificity of GPb enzyme. For these experiments, maltopentaose (G5), maltoheptaose (G7), glycogen, and CNP-G7 were used as substrates. I performed these experiments in the direction of the physiological (phosphorolytic) process, which was ensured by the 1 M phosphate content of the applied buffer. Plotting the initial reaction rates obtained from the ITC measurements as a function of the different substrate concentrations, I determined the Michaelis constant (K_M) as well as the maximal reaction rate (v_{max}). Since I did not find any kinetic data on the chain lengthening reaction of CNP-G7 in literature, I also accomplished this ITC experiment. The occurrence of the reaction in the appropriate direction was ensured by the 50 mM G-1-P content

of the applied buffer. In case of synthesis, K_M and k_{cat}/K_M became 15.2 ±2.2 mM and 0.25*10^3 $s^{-1}M^{-1}$, respectively.

Although during the phosphorolytic reaction the order of magnitude of the catalytic efficiency (k_{cat}/K_M) of the enzyme was the same in the case of the linear substrates as well as glycogen with numerous non-reducing ends, the enzyme was shown to be the most effective when using CNP-G7, since this substrate resulted in the highest k_{cat}/K_M ratio (Table 1).

Table 1: Summary of the kinetic parameters determined on various substrates, in the direction of phosphorolytic reaction catalysed by rmGPb

Substratet	Number of non-reducing ends	K_M* (mM)	k_{cat}/K_M ($s^{-1}M^{-1}$)
G5	1	20.2 ±0.3	3.6*10^3
G7	1	6.0 ±0.6	4.6*10^3
CNP-G7	1	**2.3 ±0.6**	**10.1***10^3
Glycogen	~200	1.5 ±0.1†	6.3*10^3

†: The K_M value for glycogen is given in mg/mL.
*: IC_{50} values were given as means ± fitting error.

1.3. I confirmed by ITC measurements that the substrate inhibition observed in the case of CNP-G7 could be attributed to the allosteric binding of the second substrate molecule *via* chromophore group.

When examining the substrate specificity of GPb, I observed substrate inhibition in case of the phosphorolytic reaction of the chromophore-labelled CNP-G7 substrate (Fig. 1).

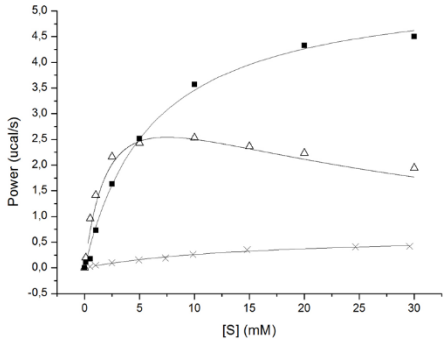


Figure 1: ITC examination of the effect of [*G7*] *and* [*CNP-G7*] *on the activity of rabbit muscle glycogen phosphorylase. Non-linear curve fittings were performed with Michaelis-Menten equation for the phosphorolysis of G7* (■) *and the synthesis of CNP-G7* (x)*, whereas with Haldane equation for the phosphorolysis of CNP-G7* (Δ)*.*

The Michaelis constant and inhibition constant (K_i) determined by Haldane equation for substrate inhibition were K_M=2.3 ±0.6 mM and K_i=23 ±7 mM. Next, I investigated the substrate inhibition induced by CNP chromophore group in the direction of phosphoryolysis with ITC method, using G7 and phosphate as substrates. As a result of this experiment, I obtained that CNP at a concentration of 9 mM is required to halve the rate of the enzyme reaction (IC_{50}=9

mM). The inhibition of CNP-G7 substrate may be explained by the fact that the G7 moiety of one substrate molecule might have been bound to the active site, while the CNP moiety of the other substrate molecule might have been bound to another binding site of the enzyme as a non-competitive inhibitor.

1.4. By determining the inhibition constants of two known glycogen phosphorylase inhibitors by ITC, I verified the suitability of the newly developed activity measurement method for performing inhibition assays.

I determined the K_i values of two known GP inhibitors (caffeine and GTH) in both directions of the reversible reaction to verify the suitability of the newly developed activity measurement method. To do this, reaction conditions had to be optimized in the first step that I performed by HPLC using CNP-G7 as a substrate. By systematically modifying the G-1-P/P_i ratio. A 10-fold excess proved to be enough to ensure the desired direction. The peak areas of the products decreased in the presence of GTH and caffeine.
For ITC experiments, I applied the natural substrate of the enzyme, namely glycogen. The occurrence of the desired reaction was ensured by the suitable G-1-P/P_i content of the buffer; 2.5 mM G-1-P/25 mM P_i ratio was utilized for phosphorolysis, whereas 25 mM G-1-P/2.5 mM P_i ratio for the glycogen chain lengthening reaction. Residual enzyme activities were plotted as a function of the different inhibitor concentrations, and then the apparent inhibition constants (K_i^*), which were determined from the non-linear regression curves fitted according to Greco-Hakala equation, were used to calculate K_i.

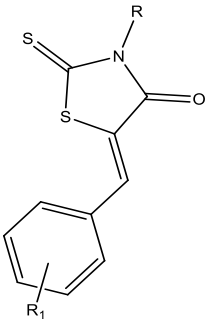
1.5. I found that for the competitive inhibitor (GTH), inhibition constants were the same in the two directions of the reversible reaction catalysed by glycogen phosphorylase. In contrast, for the compound acting via mixed-type inhibition mechanism (caffeine), the inhibition constants may differ.

Literary values obtained in the opposite directions of the GP-catalysed reversible reaction often differ markedly despite the fact that inhibition constants should be the same, since K_i corresponds to the dissociation constant of the enzyme-inhibitor complex. For caffeine, which is an inhibitor binding to the purine nucleotide binding site of GP, K_i^* was 1.9 ±0.1 mM in the direction of glycogen synthesis and 4.0 ±0.3 mM in the breakdown reaction. In the case of pure non-competitive inhibitors including caffeine, $K_i^*=K_i$. The reason for the observed divergence between the K_i values obtained for synthesis and degradation may be that caffeine is unable to bind with high affinity to GP; however, the Greco-Hakala equation used for curve fitting is primarily valid for tight binding inhibitors. K_i values resulted from the ITC analysis of GTH, which is a glucose analogue inhibitor competing with the substrate for binding to the active site of the enzyme, showed a much better agreement. K_i^* was 48.9 ±4.1 μM for the chain lengthening process and 59.9 ±3.2 μM for glycogen degradation, while the K_i values calculated from K_i^*s were 11.5 μM and 13.8 μM in the direction of glycogen synthesis and breakdown, respectively.

In the case of the competitive inhibitor (GTH), the nearly identical K_i values determined in the two opposite reactions are in good agreement with the fact that this type of inhibition is characterized by the formation of a single enzyme-inhibitor complex possessing a well-defined inhibition constant. In the case of mixed type inhibition (caffeine), however, inhibitors can bind not only to free enzymes but also to enzyme-substrate complex. Since two inhibitor complexes are then formed, two different inhibition constants are required to describe inhibition. In the GP-catalysed reversible reaction, the substrates differ in the directions of breakdown and synthesis, leading to four different inhibition constants. Even if all microscopic dissociation constants are identical in the two reactions, the resulting macroscopic constants may deviate, which may give rise to a difference in the inhibition constants obtained for each direction.

2. I revealed the molecular mechanism underlying the promiscuity of two natural and seven synthetic inhibitors with anti-hyperglycaemic activity.

The promiscuous nature and the aggregation tendency of 2-thioxo-4-thiazolidinone derivatives of synthetic origin with different substituents (Fig. 2) were investigated using indirect and direct strategies.



Number of compound	R	R_1
1	CH_2COOH	3-OCH_2CONH_2
2	CH_2COOH	4-OCH_2CONH_2
3	CH_2COOH	3-OCH_3, 4-OCH_2CONH_2
4	CH_2COOH	4-OCH_3, 3-OCH_2CONH_2
5	CH_2COOH	4-$OCH_2C_6H_5$
6	CH_2COOH	3-$OCH_2C_6H_5$
7	H	4-OC_6H_5

Figure 2: Structure of the seven examined 2-thioxo-4-thiazolidinone derivatives

The backbone of these thiazolidinones is rhodanine, which has repeatedly resulted in the promiscuous aggregation-based inhibitory properties of the developed inhibitors. For this reason, I supposed that all of the tested thiazolidinone derivatives were APIs. However, since structural modifications may allow the elimination of the factors leading to the occurrence of aggregation, I was experimentally ascertained whether the effect of 4-thiazolidinone skeleton kept having the dominance.

In the course of the indirect strategy, I examined which compounds met the criteria for APIs, i.e., the sensitivity to the presence of a detergent, enzyme-inhibitor pre-incubation, changes in enzyme concentration, and centrifugation. Using the direct strategy, I detected the presence of colloid-sized particles in solution and the changes in particle size by DLS method.

To reveal ABI, I utilized PPA as a model enzyme and $GalG_2CNP$ as a substrate.

For the experiments carried out according to the indirect strategy, I primarily used UV-VIS spectrophotometry that was supplemented with ITC measurements. When performing the direct

strategy, I used DLS method to monitor the change in the size of inhibitor and enzyme molecules over time.

Acarbose and a gallotannin were applied as control compounds. Since acarbose is a commercially available anti-diabetic agent, its specific nature was presumable, making it excellent as a negative control. As a positive control, I chose a secondary plant metabolite known as an effective α-amylase inhibitor, a tannin. Due to its polyphenolic structure as well as its several known biological effects, I had a strong suspicion that the studied gallotannin derived from Aleppo oak was a representative of APIs.

2.1 I proved the promiscuous nature of seven poorly water-soluble thiazolidinone derivatives with hydrophobic character by revealing their inhibitory effects on α-glucosidase and α-amylase enzymes.

Since one of the earliest warning signs of ABI is promiscuity, i.e., the lack of specificity, I firstly investigated whether the inhibitors are able to exert a considerable inhibitory activity on various enzymes. There are a number of publications in literature on the inhibitory effect of acarbose and gallotannins on different unrelated enzymes, thus I judged these two compounds to be promiscuous by leaning on literary data. The inhibitory activity of the seven synthetic thiazolidinone derivatives on aldose reductase and protein tyrosine phosphatase-1B enzymes was also published in literature that results became complemented with the data obtained from my inhibition studies on PPA and α-glucosidase. IC_{50} values resulted from my inhibition assays performed by spectrophotometric method and literary data are summarized in Table 2.

*Table 2: Inhibitory activity of thiazolidinone derivatives **1-7** on enzymes with diverse mechanisms and/or functions*

No. of compound	IC_{50} (μM)*			
	PPA	**α-glucosidase**	**aldose reductase[1]**	**protein tyrosine phosphatase-1B[1]**
1	194 ±3	208 ±3	0.06	717
2	96.4 ±1.7	89.6 ±2.0	0.2	max. 10% inhibition
3	96.9 ±1.8	100 ±2	0.2	378
4	66.2 ±0.3	62.1 ±1.2	0.1	679
5	294 ±5	max 21% inhibition ([I]=102 μM)	0.05	56
6	max 23% inhibition ([I]=193 μM)	141 ±5	0.05	43.1
7	abnormal behaviour	max 25% inhibition ([I]=211 μM)	0.06	63.9

*: IC_{50} values were given as means ± fitting error

Even if I consider PPA and α-glucosidase to be a single group due to their similar mechanism and function, five of the seven compounds (**1**, **3**, **4**, **5**, and **6**) can still be regarded unambiguously as promiscuous APIs due to their significant inhibitory effect on three unrelated enzymes. Although derivatives **2** and **7** met the criterion for promiscuity, i.e., they were able to inhibit multiple enzymes, the requirement for promiscuous APIs (inhibition of at least 3 significantly different enzymes) was not clearly fulfilled.

2.2 For acarbose and compounds 1, 3, 4, I proved that despite their promiscuous nature, they could not be considered as aggregation-based inhibitors.

To identify APIs by indirect strategy, I repeated the original inhibition assays (1) applying 0.01% Triton X-100 non-ionic detergent, (2) incubating the enzyme and inhibitor for 5, 10, and 30 min, (3) modifying the concentration of PPA, and (4) centrifuging the buffer solution of the inhibitor. The inhibitory activity of acarbose (negative control) as well as compounds **1**, **3** and **4** was not affected by the changed reaction conditions. Using DLS method, I confirmed and complemented my results obtained when identifying ABI according to the indirect strategy. Based on the intensity-based particle size distribution, polydispersity index, and photon count rate, I acquired direct evidence that these four compounds exerted their inhibitory activity specifically. I examined the size of inhibitor molecules as well as enzyme molecules at different time points. In the case of acarbose, no detectable particles were present even after half an hour, and no changes occurred in the size of PPA, either. However, compounds **1**, **3**, and **4** aggregated slightly (mean particle size: ~120 nm), but no alteration appeared in the size of the aggregates

[1] Maccari R, Del Corso A, Paoli P, Adornato I, Lori G, Balestri F, Cappiello M, Naß A, Wolber G, Ottanà R. Bioorganic & Medicinal Chemistry Letters. 2018; 28: 3712-3720.

over the entire measurement time. Even though inhibitor molecules underwent slight aggregation, this aggregate did not represent the active inhibitory species since the change in reaction conditions had no effect on the inhibitory activity of these three thiazolidinones. Accordingly, in the cases of these three inhibitors, I presumed a specific enzyme inhibition mechanism that involved the formation of an enzyme-inhibitor complex.

2.3 During the indirect identification of aggregation-based inhibitors, I revealed that the inhibitory effect of gallotannin and compounds 2, 5, 6, 7 was time-dependent, sensitive to detergent, centrifugation, and altering enzyme concentration. Furthermore, by dynamic light scattering measurements, I detected the presence of aggregates and that whether the inhibitor or enzyme molecules aggregated.

After investigating the fulfilment of the criteria for promiscuous APIs (sensitivity to detergent, enzyme inhibitor pre-incubation, changing enzyme concentration, and centrifugation), I proved the dependence of the inhibitory effect of gallotannin (positive control) and thiazolidinones **2**, **5**, **6** and **7** on the reaction conditions. The effect of the modifications of reaction conditions on the inhibitory activity of each inhibitor is summarized in Table 3.

*Table 3: Extent and direction of changes in the inhibitory activity of the gallotannin and thiazolidinone derivatives **2, 5, 6, 7** as a result of the modification of reaction conditions*

	Presence of detergent	**Enzyme-inhibitor pre-incubation**	**Application of multiple-fold enzyme concentration**	**Centrifugation of inhibitor solution**
Tannin	50-fold worse inhibition	4-fold better inhibition	10-fold worse inhibition	14-fold better inhibition
2	1.3-fold worse inhibition	complete cessation of inhibition	1.8-fold worse inhibition	complete cessation of inhibition
5	3.3-fold better inhibition	1.6-fold better inhibition	no change	complete cessation of inhibition
6	1.8-fold better inhibition	2.5-fold worse inhibition	1.5-fold worse inhibition	complete cessation of inhibition
7	1.4-fold better inhibition	40-fold worse inhibition	measurable inhibition	complete cessation of inhibition

Following the DLS analysis of tannin, I revealed that, contrary to the literary hypothesis that gallotannins exert their effect by inducing the aggregation of proteins, aggregated inhibitor molecules turned out to be the active inhibitory particles. A significant increase was shown in the size of tannin molecules (the monomer of 2.2 nm diameter became larger than 5000 nm within 30 minutes) while that of enzyme remained unchanged. The results obtained for thiazolidinone derivatives **2** and **6** were similar to compounds **1**, **3**, and **4**: no change could be observed in the size of the inhibitor molecules of averagely 100-200 nm even after 20 minutes. In contrast, the particle sizes of inhibitors **5** and **7** as well as the gallotannin increased significantly. Inhibitor molecules with the sizes of 509 nm ±93, 758 nm ±136, and 4886 nm ±680 were detected for compounds **5**, **7**, and the tannin, respectively. Consequently, in these three cases, the aggregation of discrete inhibitor molecules occurred. Enzyme aggregation was induced by two inhibitors: thiazolidinones **6** and **7**. After 20 minutes, three peaks appeared for

the former (peak 1: 23.5 nm ±0.8; peak 2: 70.7 nm ±3.7; peak 3: 404 nm ±9.3), while for the latter, an aggregate with a diameter of 1039 nm ±52 was present in the buffer solution of the compound.

2.4 Following the comparison of the results obtained from the measurements carried out by the direct and indirect methods, I determined the size of the aggregates, the rate of their formation, and the way of their participation in inhibitory process.

I proved that acarbose exerted its inhibitory effect through a specific, non-aggregation-based mechanism since it met neither of the criteria for APIs, and I could not detect any nano-sized particles by DLS. I drew the same conclusion for thiazolidinone derivatives **1**, **3**, and **4**, despite the fact that DLS analysis revealed the slight aggregation of these compounds.
In the case of gallotannins, I pointed out that discrete inhibitor molecules formed aggregates of thousands of nanometre, which participated in enzyme inhibition in a non-specific way, by an adsorption mechanism. Comparing the two applied methods, I found that the process of aggregation had been slow; it had taken 30 min for the aggregates of thousands of nanometre to form, which explains the considerable improvement in inhibition caused by 30 min pre-incubation of the enzyme and inhibitor (60-fold larger surface area can adsorb much more enzyme). Furthermore, based on the results obtained from the study of the effect of detergent and centrifugation, I verified that the specific binding of discrete tannin molecules could induce a greater degree of inhibition than the adsorption mechanism of the aggregate. Although considering its size, derivative **2** did not differ significantly from thiazolidinone compounds **1**, **3**, and **4**, it met all the criteria for APIs, suggesting that the aggregate should have been the active inhibitory particle. In addition, by comparing the results of the indirect and direct methods, I could conclude that this inhibitor aggregate was only able to exert its effect in the size range of approximately 63-163 nm. According to the DLS analysis of compound **5**, even a few minutes required for sample preparation were sufficient for the complete occurrence of aggregation; already in the very first measurement, I detected an aggregate with a diameter of more than 500 nm, which showed no further increase in the following 20 minutes. This result confirmed the changes observed by the direct method: after 5 minutes of enzyme-inhibitor pre-incubation, the IC_{50} value became much lower, which subsequently did not alter any further; consequently, the process of aggregation took place extremely fast in the sense of both methods. The inadequacy of the use of a 6-fold excess of enzyme might be correlated with the size of the aggregate of above 500 nm, which can ensure a particularly large adsorption surface. In the case of thiazolidinone **6**, the aggregation of protein molecules took place instead of the association of the discrete inhibitor molecules. The deterioration of inhibition due to the increase in PPA concentration as well as 30 minutes of enzyme-inhibitor pre-incubation can be attributed to the higher activity of large enzyme aggregates. For derivative **7**, the aggregate formed by the discrete inhibitor molecules could be regarded as the active particle involved in inhibitory process that colloid-sized particle induced the aggregation of the enzyme molecules. On the basis of these considerations, I drew the conclusion that all thiazolidinone derivatives

underwent a greater or lesser degree of aggregation, which was an extremely rapid process (within a few minutes required for sample preparation). The size, type, and involvement of aggregates in inhibition differed from inhibitor to inhibitor.

IV. Possible utilization of the results

The new activity measurement method developed during my doctoral work allows not only the study of the reversible reaction catalysed by glycogen phosphorylase enzyme under identical reaction conditions but also the determination of the activity and energetic background of other enzymes that also catalyse a reversible process. Using this method, the inhibition parameters obtained in the opposite directions become comparable, and it can be decided whether the results of the non-physiological studies are pharmaceutically relevant.

The other major area of my research, namely the study of the aggregation tendency of promiscuous glycoenzyme-inhibitors, has also provided a number of results that may contribute to the efficacy of the quest for antidiabetic agents by other research groups.

I demonstrated that the preliminary examination of the backbone of the compound can indeed suggest correctly the aggregation tendency of an inhibitor, which aggregation-based, non-specific inhibitory mechanism abolishes the suitability of a compound as a drug candidate. Furthermore, my experience with thiazolidinone derivatives as well as gallotannins may contribute to the complementation of the database of a software that predicts the aggregation propensity of a particular inhibitor on the basis of certain parameters and by comparing the structure of the inhibitor to be analysed with that of previously considered aggregators (e.g., *Aggregator Advisor*).

There is no agreement among the experts dealing with the investigation of drug-promiscuity with regard to that whether the degree of hydrophobicity can be related to aggregation tendency. Most publications in literature report that the more hydrophobic nature a compound has, the greater the likelihood of aggregation-based inhibitory mechanism will be. The results of my research, however, confirmed that the degree of hydrophobicity is not suitable for accurately predicting the aggregation propensity of an inhibitor. According to the *logP* values indicating the degree of hydrophobicity calculated by the *Aggregator Advisor* software, only one of the nine selected inhibitors (acarbose, gallotannin, thiazolidinone derivatives) had the chance of aggregation. Nevertheless, with the exception of acarbose, the commercially available antidiabetic drug, I revealed a greater or lesser degree of aggregation in all cases, despite the fact that some compounds had negative *logP* values, i.e., they were only slightly hydrophobic.

Finally yet importantly, I have highlighted that the literary criterion for APIs that the inhibitory activity of a compound should significantly deteriorate in the presence of a detergent is inaccurate. It would be more appropriate to say that the inhibitory activity should be altered in the presence of a detergent. This is because a detergent only results in a decrease in inhibition if the discrete inhibitor molecules induce a smaller decrease in activity than the adsorption mechanism of the aggregate.

Publications

List of publications related to the dissertation:

1. Gábor Lehoczki, Kármen Szabó, Lili Kandra, Gyöngyi Gyémánt: **Inhibition studies on α-amylase using isothermal titration calorimetry**.
Amylase, **2018**; 2: 11-16. DOI:10.1515/amylase-2018-0002

2. Kármen Szabó, Lili Kandra, Gyöngyi Gyémánt: **Studies on the reversible enzyme reaction of rabbit muscle glycogen phosphorylase b using isothermal titration calorimetry.**
Carbohydrate Research, **2019**; 477: 58-65. DOI: 10.1016/j.carres.2019.03.014 (IF (2018): 1,873)

3. Kármen Szabó, Csaba Hámori, Gyöngyi Gyémánt: **Gallotannins are Non-Specific Inhibitors of α-Amylase: Aggregates are the Active Species taking part in Inhibition.**
Chemical Biology & Drug Design, **2020**. (IF (2018): 2,256)

List of other publications:

1. Gábor Lehoczki, Kármen Szabó, István Takács, Lili Kandra, Gyöngyi Gyémánt: **Simple ITC method for activity and inhibition studies on human salivary α-amylase**.
Journal of Enzyme Inhibition and Medicinal Chemistry, **2016**; 6: 1648-1653. DOI:10.3109/14756366.2016.1161619 (IF (2018): 4,027)

List of lectures related to the dissertation:

1. Lehoczki Gábor, Szabó Kármen, Takács István, Gyémánt Gyöngyi: **Gyors ITC módszer enzimreakciók vizsgálatához.**
Doctoral Workshop, Octber 10-11, 2015, Pécs

2. Szabó Kámen, Gyémánt Gyöngyi: **Új ITC aktivitásmérési módszer a glikogén foszforiláz katalizálta reverzibilis reakció követésére.**
Professzorok az Európai Magyarországért Egyesület által szervezett XVII. PhD-Konferencia, November 15, 2018, Budapest

3. Szabó Kármen, Hámori Csaba, Gyémánt Gyöngyi: **Új ITC-alapú módszer kidolgozása a glikogén foszforiláz enzim aktivitásának méréséhez.**
I. FKF Szimpózium, April 3-5, 2019, Debrecen

4. Szabó Kármen, Gyémánt Gyöngyi: **Potenciális glikoenzim-inhibitorok aggregálódásra való hajlamának vizsgálata.**

XXII. Tavaszi Szél Konferencia, May 3-5, 2019, Debrecen
(First place in Chemical and Environmental Sciences Section, Biochemistry and Medicinal Chemistry Subsection)

List of posters related to the dissertation:

1. Erika Fazekas, Gabriella Kis, Kármen Szabó, Gyöngyi Gyémánt: **New method for the activity measurement of glycogen phosphorylase b.**
13th International Symposium and Summer School on Bioanalysis (ISSSB), June 27-July 7, 2013, Debrecen

2. Kármen Szabó, Csaba Hámori, Gyöngyi Gyémánt: **Activity and inhibition studies on rabbit muscle glycogen phosphorylase b using isothermal titration calorimetry.**
5th EFMC Young Medicinal Chemist Symposium, September 6-7, 2018, Ljubljana, Slovenia

3. Ilenia Adornato, Kármen Szabó, Jérémie Mortier, Gerhard Wolber, Rosaria Ottanà, Rosanna Maccari: **Evaluation of 4-thiazolidinone derivatives as alpha-amylase inhibitors.**
5th EFMC Young Medicinal Chemist Symposium, September 6-7, 2018, Ljubljana, Slovenia

4. Kármen Szabó, Gyöngyi Gyémánt: **Study on the aggregation tendency of known glycoenzyme inhibitors.**
13th Carbohydrate Bioengineering Meeting, May 19-22, 2019, Toulouse, France

5. Kármen Szabó, Csaba Hámori, Gyöngyi Gyémánt: **Study on the Aggregation Tendency of Tannins - or are Tannins Specific α- Amylase Inhibitors?**
7th Symposium on the Alpha-Amylase Family (ALAMY_7), September 29-October 3, 2019, Smolenice, Slovakia

6. Kármen Szabó, Gyöngyi Gyémánt: **Study on the promiscuous nature and aggregation-tendency of 4-thiazolidinone derivatives.**
21st International Conference and Exhibition on Pharmaceutics & Novel Drug Delivery Systems, March 11-12, 2020, Rome, Italy

Studies published in a peer-reviewed, edited conference volume with an ISBN number:

1. Szabó Kámen, Gyémánt Gyöngyi: **Új ITC aktivitásmérési módszer a glikogén foszforiláz katalizálta reverzibilis reakció követésére.**
Professzorok az Európai Magyarországért Egyesület, Budapest, 2018. pp. 118-128. Edited by Dr. Koncz István, Szova Ilona. ISBN 978-615-5709-05-0118-128

2. Szabó Kármen, Gyémánt Gyöngyi: **Potenciális glikoenzim- inhibitorok aggregálódásra való hajlamának vizsgálata.**

Doktoranduszok Országos Szövetsége, Budapest, 2020. pp. 105-116. Edited by Bihari Erika, Molnár Dániel, Szikszai-Németh Ketrin. ISBN 978-615-5586-61-3

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Subject: PhD Publication List

Candidate: Kármen Annamária Nagy-Szabó
Doctoral School: Doctoral School of Chemistry
MTMT ID: 10058551

List of publications related to the dissertation

Foreign language scientific articles in international journals (3)

1. **Nagy-Szabó, K. A.**, Hámori, C., Gyémánt, G.: Gallotannins are Non-Specific Inhibitors of α-Amylase: Aggregates are the Active Species taking part in Inhibition.
   Chem. Biol. Drug Des. "Accepted by Publisher", 1-28, 2020. ISSN: 1747-0277.
   IF: 2.548 (2019)

2. **Nagy-Szabó, K. A.**, Kandra, L., Gyémánt, G.: Studies on the reversible enzyme reaction of rabbit muscle glycogen phosphorylase b using isothermal titration calorimetry.
   Carbohydr. Res. 477, 58-65, 2019. ISSN: 0008-6215.
   DOI: http://dx.doi.org/10.1016/j.carres.2019.03.014
   IF: 1.841

3. Lehoczki, G., **Nagy-Szabó, K. A.**, Kandra, L., Gyémánt, G.: Inhibition studies on α-amylase using isothermal titration calorimetry.
   Amylase. 2 (1), 11-16, 2018. EISSN: 2450-9728.
   DOI: http://dx.doi.org/10.1515/amylase-2018-0002

Hungarian conference proceedings (2)

4. **Nagy-Szabó, K. A.**: Potenciális glikoenzim-inhibitorok aggregálódásra való hajlamának vizsgálata.
   In: Tavaszi Szél = Spring Wind 2019 : Tanulmánykötet. Szerk.: Bihari Erika, Molnár Dániel, Szikszai-Németh Ketrin, Doktoranduszok Országos Szövetsége, Budapest, 105-116, 2020.
   ISBN: 9786155586613

5. **Nagy-Szabó, K. A.**: Új ITC aktivitásmérési módszer a glikogén foszforiláz katalizálta reverzibilis reakció követésére.
   In: A 15 éves PEME XVII. PhD-konferenciájának előadásai. Szerk.: Koncz István, Szova Ilona, Professzorok az Európai Magyarországért Egyesület, Budapest, 118-128, 2018. ISBN: 9786155709050

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List of other publications

Foreign language scientific articles in international journals (1)

6. Lehoczki, G., **Nagy-Szabó, K. A.**, Takács, I., Kandra, L., Gyémánt, G.: Simple ITC method for activity and inhibition studies on human salivary α-amylase.
J. Enzym. Inhib. Med. Chem. 31 (6), 1648-1653, 2016. ISSN: 1475-6366.
DOI: http://dx.doi.org/10.3109/14756366.2016.1161619
IF: 4.293

Total IF of journals (all publications): 8,682
Total IF of journals (publications related to the dissertation): 4,389

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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