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Extending the investigation of 4-thiazolidinone derivatives as potential multi-target ligands of enzymes involved in diabetes mellitus and its long-term complications: A study with pancreatic α-amylase

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ABSTRACT

Diabetes mellitus is a multifactorial disease, which is frequently complicated by the development of hyperglycaemia-induced chronic complications. The therapy of diabetes mellitus often requires combinations of two or more drugs in order both to control glycaemic levels and to prevent hyperglycaemia-induced dangerous affairs. The application of multi-target agents, which are able to control simultaneously several pathogenic mechanisms, represents a useful alternative and, in fact, their discovery is a pursued aim of the research. Some (5-arylidene-4-oxo-2-thioxothiazolidin-3-yl)acetic acids, which we had previously reported as inhibitors of selected enzymes critically implicated in diabetes mellitus, were tested against pancreatic α-amylase and intestinal α-glucosidase. These enzymes catalyse the hydrolysis of dietary oligo- and polysaccharides into monosaccharides and, consequently, are responsible for postprandial hyperglycaemia; therefore, their inhibition is one of the possible strategies to control glycaemic levels in diabetes mellitus. In addition, we investigated the aggregation tendency of the tested compounds, through direct and indirect methods, in order to evaluate the mechanism of their multiple action and discover if aggregation may contribute to the inhibition of the target enzymes. Overall, compounds **1**, **3** and **4** exhibited the most favourable profile since they were shown to act as multi-target inhibitors of enzymes involved in pathways related to diabetes mellitus, without producing aggregates even at high micromolar concentrations and, therefore, can be promising agents for further developments.

1. Introduction

Diabetes mellitus (DM) has currently been one of the most outstanding and dangerous diseases of civilization. In 2019, approximately half a billion people (463 million) worldwide were affected by DM, and this number is predicted to reach 578 million and 700 million by 2030 and 2045, respectively [1]. According to the estimates of the International Diabetes Federation, last year about 4.2 million people aged 20–79 years died as a consequence of DM and its complications [2]. In particular, hyperglycaemia-induced long-term complications, such as retinopathy, nephrophathy, neuropathy, and cardiovascular diseases, contribute to this high mortality rate [3].

In spite of the availability of numerous drugs for the regulation of hyperglycaemia, there has been growing demand for the further design and synthesis of potential agents for the treatment of DM and its associated pathologies [4,5]. One possible way to control these pathological states is to inhibit the activity of enzymes involved in carbohydrate metabolism and in the regulation of insulin signalling (e.g. aldose reductase, protein phosphatases, α-amylase, α-glucosidase, etc.) [6–10].

Aldose reductase (AR; EC 1.1.1.21), which belongs to the group of oxidoreductases, catalyses the reduction of glucose to sorbitol that is the first step of polyol pathway of glucose metabolism [11]. Under hyperglycaemic conditions, as in DM, increased metabolism of glucose through the polyol pathway results in osmotic alterations and redox imbalance that trigger a cascade of dangerous events responsible for vascular and nervous lesions and, ultimately, the onset and progression of diabetic long-term complications [10,11]. Protein tyrosine phosphatase 1B (PTP1B; EC 3.1.3.48) plays a key role in the negative regulation of protein tyrosine phosphorylation in the insulin signalling cascade by dephosphorylating specific tyrosine residues of insulin receptor and its

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substrates; therefore its inhibition may contribute both to improve insulin action and to counteract insulin-resistance in diabetic patients [9,12].

In addition, postprandial hyperglycaemia, which is the hallmark of DM, may be correlated with the activities of pancreatic α-amylase and intestinal α-glucosidase enzymes as these enzymes are mainly responsible for the breakdown of oligo- and polysaccharides into monosaccharides. The former enzyme catalyses the cleavage of alpha bonds in starch or glycogen to yield dextrin, maltose, or maltotriose, whereas the latter is in charge of the further hydrolyses of these carbohydrates into glucose. Therefore, the inhibition of all these enzymes may be regarded as a significant strategy in the management of DM [13,14].

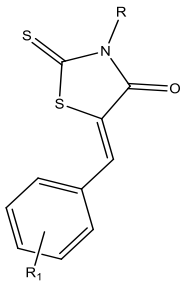
Moreover, 'multi-target ligands' (MTL) have recently been designed and synthetized, which can provide potentially better therapeutic effects than a single-target drug [15–17]. Therapeutic application of rationally designed MTL could be more advantageous than combination of highly selective single-target medicaments since it could prevent drug-drug-interactions or different pharmacokinetic behaviour, and it could lead to better patient compliance. The therapeutic management of complex diseases, such as DM, could particularly benefit from multi-target drugs capable of controlling several pathogenesis simultaneously [18].

4-Thiazolidinone can be considered as a "privileged" structure in medicinal chemistry research, since it can be associated with many different biological effects, such as antibacterial, anti-inflammatory, antineoplastic, and antidiabetic activities. In particular, in the context of antidiabetic drugs, the 2,4-thiazolidindione pioglitazone is a well-known oral drug used for the management of type 2 DM, as well as epalrestat, which is a 2-thioxo-4-thiazolidinone derivative, is the only drug approved for the treatment of diabetic neuropathy. In addition, some 4-thiazolidinones have recently been found to be able to inhibit α-amylase and α-glucosidase [19,20].

In recent years, some of us have been dealing with the design, synthesis, and structure-activity relationship (SAR) study of 4-thiazolidinone derivatives that are potential inhibitors of enzymes responsible for the development of type 2 DM as well as its long-term complications, such PTP1B and AR. We found that a (2-oxo/thioxo-4-thiazolidin-3-yl) acetic acid moiety is important to achieve potent AR inhibitory effects, allowing the identification of inhibitors endowed with low submicromolar IC_{50} values towards bovine and human AR enzymes [10,21,22]. In addition, 4-[(5-arylidene-4-thiazolidin-3-yl)methyl]benzoic acid derivatives were shown to be good inhibitors of human PTP1B, also with promising insulin-mimetic effects in cellular cultures [23–27]. On the whole, specific SARs and action mechanisms were defined, highlighting that the substituents present in the positions 2, 3, and 5 of the thiazolidine scaffold can differently influence the inhibitory activity towards various targets. More recently, starting from the SARs acquired so far, 4-thiazolidinone derivatives were synthesized and evaluated as dual inhibitors for both AR and PTP1B, resulting in the identification of compounds displaying potent inhibitory activity towards AR along with moderate effects against PTP1B [28]. Now we decided to extend the study of some (5-arylidene-4-oxo-2-thioxothiazolidin-3-yl)acetic acid representatives (compounds **1**–**7**, Table 1) by evaluating their activity against additional enzymes involved in DM, such as pancreatic α-amylase (PPA) and α-glucosidase.

In addition, in order to better delineate the potential applicability of these multi-target candidates, we also assessed their aggregation tendency. In fact, adverse reactions may emerge as a consequence of the formation of colloidal aggregates of drug candidates in aqueous environment. Such aggregation-prone compounds may exert their inhibitory effects in two ways: (1) discrete inhibitor molecules may aggregate into particles up to 1000 nm in diameter, thus interacting with a specific part of the target protein [29,30] or (2) the non-specific adsorption of protein molecules onto the surface of the aggregate particles may lead to the modulation of enzyme activity due to the partial unfolding of protein [31,32]. Therefore, aggregation-based promiscuous compounds are often capable of inhibiting enzyme activity *via* non-specific interactions [33,34].

Table 1
Structures of (5-Arylidene-4-oxo-2-thioxothiazolidin-3-yl)acetic acids **1**–**7**.



Comp.	R	R1
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

We applied both direct and indirect methods to evaluate the tendency of inhibitors to aggregate. Direct strategies leaned on the determination of particle sizes measured using dynamic light scattering (DLS), whereas indirect detection of aggregation involved the investigation of inhibitory process in terms of time-dependence, sensitivity to the presence of a non-ionic detergent, and the effect of the change in enzyme concentration [35,36]. Within the meaning of the indirect strategy, a compound can be regarded as promiscuous aggregation-based inhibitor (ABI) if the following conditions are satisfied: (1) reduced inhibitory activity in the presence of a non-ionic detergent, (2) significant inhibitory effect on at least three unrelated enzymes, (3) diminished inhibition as a result of a multiple-fold increase in protein concentration, and (4) enhanced inhibition with enzyme-inhibitor pre-incubation [37,38].

2. Experimental section

2.1. Materials

The α-amylase inhibition assay was performed applying 2-chloro-4-nitrophenyl 4-*O*-β-D-galactopyranosyl-α-maltoside ($GalG_2CNP$) as substrate and porcine pancreatic α-amylase (PPA) as enzyme. $GalG_2CNP$ was purchased from SORACHIM SA (Lausanne, Switzerland), whereas PPA (type 6B) was obtained from Sigma-Aldrich (St. Louis, MO, USA). We had dialyzed commercial amylase powder using Amicon 30 K filter device (Millipore, MA, USA) to remove its salt content. The concentration of the purified enzyme stock solution was 28 mg/mL, which was determined with Bradford method [39]. The enzyme and substrate were dissolved in 50 mM MES buffer (pH 6.0), containing 5 mM $Ca(OAc)_2$, 51.1 mM NaCl, and 152 mM sodium azide, as proposed for the activity measurements carried out with $GalG_2CNP$ substrate [40].

The inhibition of α-glucosidase activity was carried out using *Saccharomyces cerevisiae* α-glucosidase (type I, Sigma-Aldrich, St. Louis, MO, USA) as enzyme, 10 mM *p*-nitrophenyl-α–D-glucopyranoside (PNP-α-Glc) as substrate (Sigma-Aldrich, St. Louis, MO, USA). The concentration of the enzyme stock solution was 1.5 mg/mL. The enzyme and substrate were dissolved in 0.2 M of Na-phosphate buffer (pH 6.8), containing 0.2 M $Na_2HPO_4{\cdot}H_2O$ and 0.2 M $NaH_2PO_4{\bullet}\ 2H_2O$ [41].

2-thioxo-4-thiazolidinone inhibitors were synthetized following a

procedure that we previously reported [28]. Since these compounds are poorly soluble in water, dimethyl sulfoxide (DMSO) was used for the preparation of inhibitor stock solutions as well as serial dilutions. DMSO was obtained from Molar Chemicals Ltd (Halásztelek, Hungary). Acarbose was extracted from Glucobay® tablets (Bayer, Leverkusen, Germany) and the purity analysis was carried out using positive-ion reflectron MALDI-TOF MS.

Sigma Aldrich (St. Louis, MO, USA) provided all of the components involved in the applied buffer, whereas Triton™ X-100 non-ionic detergent was purchased from Reanal Laboratory Chemicals Ltd (Budapest, Hungary).

2.2. Methods

2.2.1. UV/VIS spectrophotometric analysis

We used a Jasco V550 double-beam UV/VIS Spectrophotometer (Jasco Corporation, Easton, MD, USA) for the examination of promiscuity and aggregation-tendency of 2-thioxo-4-thiazolidinones and acarbose. Substrates and enzymes were dissolved in the appropriate buffer. In the case of thiazolidinones, a total of 500 μL reaction mixture containing 460 μL of buffer (phosphate buffer, pH 6.8, for α-glucosidase assay; MES buffer, pH 6, for α-amylase assays), 20 μL of 10 mM PNP-α-Glc or 5 mM $GalG_2CNP$ substrate dissolved in buffer, 10 μL of enzyme (1.5 mg/mL α-glucosidase, 28 μg/mL PPA), and 10 μL of inhibitor dissolved in DMSO was thoroughly mixed. For acarbose, the reaction mixture was prepared in a final volume of 400 μL containing 80 μL of 5 mM $GalG_2CNP$ substrate, 10 μL of inhibitor, and 300 μL of 50 mM MES buffer. The reaction was started by adding a 10 μL aliquot of 112 μg/mL PPA to the sample.

We measured the initial rate (v_0) of enzyme reaction by monitoring the increase in absorbance induced by the release of chromophore group (CNP or PNP) at 400 nm for 180 s. Thereafter, the slope of the initial phase of the curve was determined with the use of Time Course Measurement function of Jasco Spectra Manager™ Software (Jasco Corporation, Easton, MD, USA). We compared these *ΔAbs*/min values, corresponding to v_0, to the value resulting from the measurement of the uninhibited enzyme activity in order to calculate the residual enzyme activity (in percentage) for each inhibitor concentration. IC_{50} values were determined on the basis of the concentration-response curves obtained by plotting the residual activities as a function of the inhibitor concentrations. Logistic curves were fitted using non-linear regression implemented in the Microcal Origin 7.0 scientific graphing and data analysis software (Malvern, Worcestershire, UK).

2.2.1.1. Examination of the sensitivity to the presence of detergent. The investigation of the sensitivity of the inhibitors to a detergent and the evaluation of the results were performed in the same manner as described above, except that MES buffer used to dissolve the enzyme, substrate and inhibitors contained 0.01% or 0.1% Triton X-100 non-ionic detergent.

2.2.1.2. Examination of the effect of enzyme-inhibitor pre-incubation. To study the time-dependent inhibitory effect, PPA and inhibitors were incubated for 5, 10, and 30 min, and then the enzyme reaction was initiated by adding a 20 μL aliquot of $GalG_2CNP$ substrate to the sample. When examining the sensitivity of the thiazolidinone derivatives and acarbose to pre-incubation, we applied the reaction mixture in the same composition as before.

2.2.1.3. Examination of the sensitivity to the change in enzyme concentration. When we examined the effect of the change in enzyme concentration, we were able to apply a maximum of 6-fold protein concentration due to the extremely fast enzyme reaction resulting from the excessive activity of uninhibited PPA. These measurements were performed by initiating the reaction with 5, 20, and 30 μL of enzyme instead of 10 μL of PPA used in previous inhibition assays. The volume of buffer was modified so that the enzyme-buffer volume ratio remained unchanged.

2.2.1.4. Inhibition assay followed by centrifugation. We repeated the inhibition assay after centrifuging the test compound in MES buffer. 20 μL of inhibitor in 980 μL of assay buffer was centrifuged at 14,000 g for 30 min at room temperature. Then we added 20 μL of substrate and 10 μL of enzyme to 470 μL of the supernatant removed from the centrifuged sample [34]. Subsequently, we determined the IC_{50} value by using the same spectrophotometric manner described above.

2.2.2. Dynamic light scattering (DLS) analysis

To determine the particle sizes of 2-thioxo-4-thiazolidinones and acarbose, we applied a ZetaSizer Nano ZS DLS device equipped with a 4 mW He–Ne laser operating at a wavelength of 633 nm and a detection angle of 173° (Malvern Panalytical Ltd, Worcestershire, UK). Each measurement involved three sub-runs with a 30 s duration for each run. 1 mL of sample was analyzed at 37 °C using disposable polystyrene cuvettes. Prior to each measurement, we purified the inhibitor solutions, the enzyme solution as well as the buffer itself by filtering them on 0.22 μm membranes (Millex®-GP units) purchased from Millipore™ (Massachusetts, USA) to avoid the interferences caused by the presence of contaminations. Two sets of experiment were performed by DLS: first, 20 μL of inhibitor (at the concentration close to its IC_{50}) dissolved in DMSO was added to 980 μL of MES buffer, and this solution became analyzed; secondly, the sample contained 20 μL of inhibitor dissolved in DMSO (at the concentration close to its IC_{50}), 20 μL of PPA enzyme in the buffer (0.56 mg/mL), and 960 μL of MES buffer (pH 6).

We applied Malvern DTS 7.12 software to collect and analyse the obtained data. All particle sizes stated in this paper are based on mean intensities. (Intensity PSD values: intensity based particle size distribution). We chose intensity based particle size distribution (Intensity PSD), which correlates to the amount of light scattered by the particles, since this kind of distribution provides the most reliable value, especially for polydisperse samples. Furthermore, intensity PSD does not require any previous knowledge of the sample properties (e.g. refractive index, absorption, etc.) compared to other types of particle size distribution values (e.g. size distribution by number or volume) [42].

3. Results

3.1. Study on the multi-target activity of (5-arylidene-4-oxo-2-thioxothiazolidin-3-yl)acetic acids 1-7

As a first step, we investigated the inhibitory effects of 2-thioxo-4-thiazolidinone derivatives **1**–**7** towards PPA and α-glucosidase. We recently reported the inhibitory activities of these 4-thiazolidinone derivatives towards human AR and PTP1B [28].

Each of the tested compounds **1**–**7** exhibited potent inhibitory effects towards human AR, representing submicromolar/nanomolar IC_{50} values, whereas they proved to be only moderate inhibitors of human PTP1B since only compounds **5**–**7** possessed appreciable inhibitory properties at concentrations <100 μM (Table 2) [28].

When tested against PPA (EC 3.2.1.1) and α-glucosidase (EC 3.2.1.20), compounds **1**–**7** exhibited diverse inhibitory potencies, ranging from considerable effects at concentrations ≤ 100 μM (compounds **2**–**4**) to modest or negligible activity (compounds **1**, **5**–**7**) (Table 2). In the case of compound **7**, although it inhibited AR and PTP1B with low submicromolar and micromolar IC_{50} values, respectively, only 25% inhibition was achieved (at 211 μM concentration) towards α-glucosidase, whereas the IC_{50} against PPA was not defined since the continuously lowering absorbance did not allow us the determination of *ΔAbs*/min values. We labelled this phenomenon as 'abnormal behaviour'.

Table 2
Inhibitory activity of compounds **1**–**7** against different enzymes, expressed as IC_{50} (μM) or inhibition percentage at the indicated concentration.

Comp.	R	R_1	IC_{50} (μM)[a]			
			PPA	α-glucosidase	AR[b]	PTP1B[b]
Acarbose	–	–	18.1 ± 0.1[c]	0.28 ± 0.02[d]	–	–
1	CH_2COOH	3-OCH_2CONH_2	194 ± 3	208 ± 3	0.064 ± 0.005	717 ± 136
2	CH_2COOH	4-OCH_2CONH_2	96.4 ± 1.7	89.6 ± 2.0	0.194 ± 0.011	10%[e]
3	CH_2COOH	3-OCH_3, 4-OCH_2CONH_2	96.9 ± 1.8	100 ± 2	0.228 ± 0.012	378 ± 23
4	CH_2COOH	4-OCH_3, 3-OCH_2CONH_2	66.2 ± 0.3	62.1 ± 1.2	0.139 ± 0.011	679 ± 184
5	CH_2COOH	4-$OCH_2C_6H_5$	294 ± 5	21%[f]	0.052 ± 0.003	56.0 ± 1.0
6	CH_2COOH	3-$OCH_2C_6H_5$	21%[g]	141 ± 5	0.053 ± 0.004	43.1 ± 1.5
7	H	4-OC_6H_5	a.b[h].	25%[i]	0.060 ± 0.004	63.9 ± 2.0

[a] IC_{50} values were given as means ± fitting error
[b] Ref. [28]
[c] Ref. [43]
[d] Ref. [44]
[e] inhibition percentage at 300 μM concentration Ref.
[f] inhibition percentage at 102 μM concentration
[g] inhibition percentage at 193 μM concentration
[h] abnormal behaviour
[i] inhibition percentage at 211 μM concentration

Results obtained from our current and previous studies showed that some of the tested compounds, in particular analogues **1**, **3**, **4**, **5** and **6**, appear to be capable of exerting their inhibitory effects on three unrelated enzymes (accounting PPA and α-glucosidase as a single group since they have similar mechanisms and functions). However, in several cases, the inhibitory potencies observed for the different enzymes, are modest. Nonetheless, we further investigated these compounds to evaluate if their action might be originated from specific interactions with multiple targets or from non-specific aggregation-based inhibition.

3.2. Investigation of the aggregation-tendency of 4-thiazolidinone derivatives

In the subsequent step, we used *Aggregator Advisor* tool to identify those 4-thiazolidinones that might prone to aggregation [45]. Considering their chemical structures and physical properties, the program did not find any similarities to known aggregators. Furthermore, only one of the selected seven derivatives (compound **7**) had a comparatively high calculated *logP* value (3.6), which falls in the range reported for many other aggregators. In all other cases, calculated *logP* values did not exceed the limit (*logP* > 3) described in literature for aggregation-prone compounds [46]. *LogP* values provided by *Aggregator Advisor* for the remaining inhibitors are as follows: 0.1 for compounds **1** and **2**, -0.5 for compounds **3** and **4**, finally 2.8 for compounds **5** and **6**. In any case, it may be not sufficient to consider the calculated *logP* value as a predictive parameter since recent studies proved that a number of drugs on market or in clinical trials have *clogP* higher than 3, and several MTLs with significant differences in their lipophilicity can be found at various therapeutic fields [47,48].

In order to assess their action mode, we relied on both indirect and direct strategies. The indirect strategy involved the investigation of the fulfilment of the main criteria for aggregators (time-dependence, sensitivity to the presence of a non-ionic detergent, and altered inhibitory potency due to the change in the enzyme concentration as well as centrifugation), whereas the direct strategy included the measurement of particle sizes by DLS. We applied acarbose in these experiments as a negative control, since it is a commercially available anti-diabetic agent, with a known specific non-aggregating inhibitory mechanism towards α-glucosidase.

3.2.1. Sensitivity of the inhibitory activity of 4-thiazolidinone derivatives to the presence of detergent

In order to distinguish the ABIs from non-aggregation-based inhibitors, we checked their sensitivity to the presence of a non-ionic detergent. For this purpose, we repeated the inhibition assay with the use of MES buffer containing 0.01% Triton X-100. Similar to acarbose, the application of non-ionic detergent did not influence the IC_{50} values of compounds **1, 3, 4**; a maximum of 3% change occurred (Table 3; see Fig. S1 in Supplementary Material for the fitted curves).

In the case of derivative **2**, mild loss of inhibition was observed in the presence of 0.01% Triton X-100; its original IC_{50} value increased by 12%. Since this growth was not conclusive, we repeated the experiment using a higher detergent content (0.1%). The obtained results suggested that we might be faced with aggregation-based inhibition since the inhibitory activity of compound **2** decreased by approximately 32% as a result of the inclusion of 0.1% Triton X-100 (Table 3).

The results obtained for compounds **5**–**7** indicated an opposite behaviour, in fact, although considerable changes occurred in the inhibitory parameters of these compounds, an improved inhibitory effect appeared instead of the expected deterioration (Table 3).

Table 3
IC_{50} values for compounds **1**–**7** towards PPA obtained in the presence and absence of Triton X-100 non-ionic detergent.

Comp.	R	R_1	IC_{50} (μM)[a]		
			- detergent	+0.01% detergent	+0.1% detergent
Acarbose	–	–	0.65 ± 0.02	0.67 ± 0.01	–
1	CH_2COOH	3-OCH_2CONH_2	194 ± 3	192 ± 4	–
2	CH_2COOH	4-OCH_2CONH_2	96.4 ± 1.7	108 ± 2	127 ± 1
3	CH_2COOH	3-OCH_3, 4-OCH_2CONH_2	96.9 ± 1.8	93.9 ± 0.5	–
4	CH_2COOH	4-OCH_3, 3-OCH_2CONH_2	66.2 ± 0.3	67.3 ± 0.9	–
5	CH_2COOH	4-$OCH_2C_6H_5$	294 ± 5	149 ± 4	88.9 ± 0.7
6	CH_2COOH	3-$OCH_2C_6H_5$	23%[b]	33%[b]	41%[b]
7	H	4-OC_6H_5	a.b.[c]	109 ± 1	81.3 ± 1.9

[a] IC_{50} values were given as means ± fitting error.
[b] Inhibition percentage at 193 μM concentration;[c] abnormal behaviour.

3.2.2. Time-dependence of the inhibitory activity of 4-thiazolidinone derivatives

To investigate the following indirect indication of aggregation, namely the time-dependent inhibitory activity of compounds **1**–**7**, we pre-incubated PPA with each inhibitor for 5 min, 10 min, and 30 min before the initiation of enzyme reaction by the addition of substrate. Just as in the case of acarbose used as a negative control, IC_{50} values remained unchanged for compounds **1, 3,** and **4** in each of the applied pre-incubation periods, whereas for derivatives **2**, **6**, and **7**100%, 14%, and 28% loss of inhibition were observed, respectively, following the 30-min pre-incubation (see Fig. S2 in Supplementary Material for the fitted curves). A general feature of aggregation-based inhibition is the increasing inhibitory effect as a result of prolonged incubation time. This time-dependent behaviour was observed only for compound **5**, which displayed a gain in potency of 38% after 5 min pre-incubation; in fact, its IC_{50} value was 1.6-fold lower than that observed without incubation, and this improved activity remained unaltered throughout the following 30 min (Table 4).

3.2.3. Sensitivity of the inhibitory activity of 4-thiazolidinone derivatives to the change in enzyme concentration

For examining the last criterion for aggregation-tendency, namely the sensitivities of the tested compounds to the changes in enzyme concentration, we repeated the inhibition assays by applying PPA at different concentrations. As observed for acarbose, increasing the amount of enzyme did not have a noticeable effect on the inhibitory activity of compounds **1, 3, 4**, and **5** (Table 5; see Fig. S3 in Supplementary Material for the fitted curves). Mitigated modification could be observed in the inhibitory efficiency of derivative **6**; its maximal available inhibition deteriorated from 27% to 19% by raising the enzyme concentration from 0.23 μg/mL to 1.68 μg/mL. The most outstanding difference was obtained in the case of compound **2**, which displayed an IC_{50} value 1.8-fold higher when we applied the enzyme in a 6-fold higher concentration (Table 5).

3.2.4. Sensitivity of the inhibitory activity of 4-thiazolidinone derivatives to centrifugation

An additional indirect strategy was applied for confirming or excluding the aggregation of 4-thiazolidinone derivatives. In this assay, we repeatedly determined the IC_{50} values for each of the inhibitors after the centrifugation of the mixture of the test compound and the assay buffer. As a result of the centrifugation, compounds **2**, **5**, **6**, and **7** completely lost their inhibitory activities, whereas the degree of inhibition suffered almost no changes in the case of derivatives **1**, **3**, and **4** (Table 6).

Table 5
Effect of the change in enzyme concentration on the inhibitory activities of compounds 1–7 towards PPA.

Comp.	R	R_1	IC_{50} (μM)[a]			
			[E] = 0.28 μg/mL	[E] = 0.56 μg/mL	[E] = 1.12 μg/mL	[E] = 1.68 μg/mL
Acarbose	–	–	0.71 ± 0.03	0.65 ± 0.02	0.71 ± 0.03	0.68 ± 0.04
1	CH_2COOH	3-OCH_2CONH_2	198 ± 2	194 ± 3	191 ± 2	197 ± 2
2	CH_2COOH	4-OCH_2CONH_2	89.1 ± 3.2	96.4 ± 1.7	119 ± 3	163 ± 4
3	CH_2COOH	3-OCH_3,4-OCH_2CONH_2	95.6 ± 1.0	96.9 ± 1.8	89.9 ± 0.8	94.2 ± 0.8
4	CH_2COOH	4-OCH_3,3-OCH_2CONH_2	67.1 ± 1.4	66.2 ± 0.3	68.3 ± 0.8	68.5 ± 1.4
5	CH_2COOH	4-$OCH_2C_6H_5$	290 ± 14	294 ± 5	295 ± 12	290 ± 10
6	CH_2COOH	3-$OCH_2C_6H_5$	27%[b]	23%[b]	19%[b]	19%[b]
7	H	4-OC_6H_5	a.b.[c].	a.b.[c]	a.b.[c]	39.5%[d]

[a] IC_{50} values were given as means ± fitting error.
[b] Inhibition percentage at 193 μM concentration.
[c] Abnormal behaviour.
[d] Inhibition percentage at 119 μM concentration.

Table 6
Effect of 30-min centrifugation on the inhibitory activities of compounds **1**–**7** towards PPA.

Comp.	R	R_1	IC_{50} (μM)[a]	
			without centrifugation	after 30 min centrifugation
1	CH_2COOH	3-OCH_2CONH_2	194 ± 3	200 ± 5
2	CH_2COOH	4-OCH_2CONH_2	96.4 ± 1.7	n.i.[b]
3	CH_2COOH	3-OCH_3, 4-OCH_2CONH_2	96.9 ± 1.8	105 ± 2
4	CH_2COOH	4-OCH_3, 3-OCH_2CONH_2	66.2 ± 0.3	65.6 ± 1.6
5	CH_2COOH	4-$OCH_2C_6H_5$	294 ± 5	n.i.[b]
6	CH_2COOH	3-$OCH_2C_6H_5$	23%[c]	n.i.[b]
7	H	4-OC_6H_5	a.b.[d]	n.i.[b]

[a] IC_{50} values were given as means ± fitting error.
[b] No inhibition.
[c] Inhibition percentage at 193 μM concentration.
[d] Abnormal behaviour.

Table 4
Summary of the IC_{50} values for compounds **1**–**7** following 0 min, 5 min, 10 min, and 30 min enzyme-inhibitor pre-incubation.

Comp.	R	R_1	IC_{50} (μM)[a]			
			without pre-incubation	5 min pre-incubation	10 min pre-incubation	30 min pre-incubation
Acarbose	–	–	0.65 ± 0.02	0.64 ± 0.02	0.64 ± 0.03	0.65 ± 0.04
1	CH_2COOH	3-OCH_2CONH_2	194 ± 3	195 ± 2	201 ± 3	200 ± 3
2	CH_2COOH	4-OCH_2CONH_2	96.4 ± 1.7	13%	n.i.[b]	n.i.[b]
3	CH_2COOH	3-OCH_3, 4-OCH_2CONH_2	96.9 ± 1.8	94.5 ± 1.3	97.0 ± 1.6	96.6 ± 1.5
4	CH_2COOH	4-OCH_3, 3-OCH_2CONH_2	66.2 ± 0.3	69.0 ± 1.6	68.9 ± 2.1	69.7 ± 1.1
5	CH_2COOH	4-$OCH_2C_6H_5$	294 ± 5	181 ± 9	189 ± 4	186 ± 8
6	CH_2COOH	3-$OCH_2C_6H_5$	23%[c]	18%[c]	12%[c]	9%[c]
7	H	4-OC_6H_5	a.b.[d]	108 ± 2	114 ± 3	151 ± 5

[a] IC_{50} values were given as means ± fitting error.
[b] No inhibition.
[c] Inhibition percentage at 193 μM concentration.
[d] Abnormal behaviour.

3.2.5. Dynamic light scattering analysis

According to the above-described experiments, compounds **1, 3,** and **4** were shown to be specific MTL since they did not display aggregation behaviour, and hence we can exclude a non-specific inhibition mechanism for these compounds. In the case of analogues **2**, **5**, **6**, and **7**, our data are not always consistent with the properties of aggregators and only some results suggested that they might be likely non-specific ABIs.

Therefore, we chose a direct way, namely the use of DLS for the analysis of aggregate formation. The change in the particle sizes of 4-thiazolidinones and PPA was monitored over time. We applied the inhibitors at the concentration close to their IC_{50} towards PPA and measured the size of the inhibitor as well as that of PPA at five different times (0 min, 2 min, 5 min, 10 min, and 20 min). As expected, no detectable particles were present in the aqueous solution of acarbose, and the size of the enzyme remained unaltered during the whole measurement. Although we could detect particles (~100 nm on average) for derivatives **1**, **3** and **4**, they did not fall in the range characteristic of aggregates, moreover, no tendentious changes occurred in time. In contrast, considerably large particles were shown in the case of compounds **2**, **5** and **7** (Table 7), whereas for compound **6**, a significant change in the size of enzyme molecules could be observed. In the very first measurement for compound **6** (immediately after mixing PPA and the inhibitor), we obtained only one peak belonging to the protein of original size (121 nm ± 5 at 0 min); however, 20 min after mixing these two components, three peaks were detected by DLS (1. peak: 23.5 nm ± 0.8, 2. peak: 70.7 nm ± 3.7, 3. peak: 404 nm ± 9.3). Furthermore, derivative **7** resulted in a unique outcome: the growth in the size of inhibitor molecules was accompanied by an notable rise in the size of enzyme molecules; their size became an order of magnitude higher (1039 nm ± 52) in comparison with the size of PPA measured at 0 min (156 nm ± 32). In all other cases, the sizes of both the inhibitors and the protein molecules remained nearly unchanged during the investigation.

4. Discussion

When we investigated thiazolidinones **1**–**7** as inhibitors of some unrelated enzymes (AR, PTP1B, PPA, α-glucosidase), which are all implicated in the development of DM and its long-term complications, five of them (compounds **1**, **3**, **4**, **5**, and **6**) were found to be capable of inhibiting the selected enzymes at significantly different concentrations.

Since non-specific aggregation-based inhibition is frequently observed, and aggregating behaviour has been detected even among marketed drugs that act on specific targets [35], we decided to further investigate these MTLs to evaluate whether they were non-specific ABIs.

When, prior to our experiments, we attempted to predict which one of the 4-thiazolidinones might be prone to aggregation, we could make some observations. According to *Aggregator Advisor*, only compound **7** was assumed to be liable for aggregation considering its calculated *logP* exceeding the limiting value for aggregators. However, it is worth noting that many MTLs available or candidate as drugs have higher *logP* values, despite not being aggregators. On the other hand, although 2-thioxo-4-thiazolidinone derivatives have gained a reputation as potentially problematic compounds prone to act with indiscriminate mechanisms, it has been demonstrated that their promiscuous behaviour is often originated from the numerous possible specific interactions between the substituents of the heterocyclic scaffold and the biological targets [49]. Therefore, it is recommended to establish their mode of action, without excluding them *a priori* from further development due to their potential value as bioactive molecules.

When derivatives **1**–**7** were tested towards PPA, compounds **1**, **3**, and **4** proved to be promising agents since they were shown to be non-ABIs. Even though we were able to detect particles of about 100 nm by DLS, neither the size of the inhibitor molecules or that of enzyme molecules increased over time. Furthermore, examining the main criteria for ABIs, we did not observe any significant alterations in their inhibitory efficiencies. In fact, the IC_{50} values of compounds **1**, **3** and **4** remained unchanged after the application of Triton X-100 non-ionic detergent and 6-fold higher enzyme concentration as well as following the enzyme-inhibitor pre-incubation. In addition, there were no evidences of the presence of aggregates even subsequent to the centrifugation of the inhibitor solutions for 30 min.

Despite the fact that in the case of compounds **2**, **5**, **6**, and **7**, some of the obtained results are not entirely consistent with the behaviour characteristic of ABIs, they are likely to exert their effect *via* aggregation as they completely lost their inhibitory activity against PPA after centrifugation, and, in addition, they complied some other criteria for aggregators. The inhibitory effect of derivative **2** against PPA was slightly attenuated in the presence of 0.01% Triton X-100 non-ionic detergent, since its IC_{50} value increased only by 12%, whereas the inhibitory potencies of most aggregators undergo at least a twofold decrease [50,51]. Therefore, we repeated this experiment by using the detergent at higher concentration (0.1%). In the latter case, the IC_{50} further increased to 127 μM ± 1, which represents a fall of 32% in the inhibitory potential of compound **2**. This suggests that derivative **2** may act *via* aggregation since a detergent can diminish or even totally prevent the formation of aggregates (regardless of whether the inhibitor or protein molecules form aggregates) by reducing non-specific interactions, and hence an ABI cannot exert its full effect on the target enzyme. Although Triton X-100 could form micelles, especially at concentrations higher than 0.01%, potentially able to incorporate molecules also of specific inhibitors preventing thus their interaction with the target [35], the fulfilment of other criteria appeared to confirm the assumption that compound **2** might act as an aggregator. In fact, the IC_{50} value of compound **2** increased by about 82% (1.8-fold) with the application of 6-fold higher enzyme concentration, suggesting that the inhibitor molecules themselves should be aggregated. This is due to the fact that discrete inhibitor molecules can adsorb enzyme molecules only in a specified ratio [29]. Therefore, following the complete saturation of the aggregated inhibitor particles, they will not able to adsorb any additional enzyme molecules, thus, the concentration of free enzyme molecules with uninhibited activity will be higher. However, in contrast, when we investigated the effect of enzyme-inhibitor pre-incubation, the inhibitory effect of compound **2** towards PPA decreased until it disappeared. According to the principle reported in literature, the more time we ensure, the larger aggregate will be formed, and hence the more enzyme molecules will be adsorbed on the surface of the aggregates, resulting in the enhancement of the inhibitory activity [30,37]. However, we observed just the opposite, and after 10-min pre-incubation of compound **2** and PPA, no inhibition could be detected at the highest applied concentration (209 μM). The aggregation of molecules of compound **2** was also confirmed by the centrifugation counter-screen as well as DLS measurements. In the former case, derivative **2** completely lost its

Table 7
Intensity-normalized diameters of compounds **1**–**7** determined at five different times.

	Comp. 1 (204 μM)	Comp. 2 (105 μM)	Comp. 3 (103 μM)	Comp. 4 (73.9 μM)	Comp. 5 (208 μM)	Comp. 6 (193 μM)	Comp. 7 (137 μM)
0 min	153 nm ± 37	63 nm ± 6	125 nm ± 42	167 nm ± 52	506 nm ± 104	82 nm ± 12	201 nm ± 75
2 min	167 nm ± 59	163 nm ± 47	143 nm ± 56	167 nm ± 64	520 nm ± 101	97 nm ± 21	599 nm ± 103
5 min	165 nm ± 62	191 nm ± 34	129 nm ± 39	176 nm ± 80	532 nm ± 129	124 nm ± 38	679 nm ± 116
10 min	152 nm ± 44	240 nm ± 81	152 nm ± 22	182 nm ± 77	529 nm ± 132	103 nm ± 24	700 nm ± 126
20 min	167 nm ± 49	236 nm ± 80;	196 nm ± 40	195 nm ± 91	509 nm ± 93	110 nm ± 28	758 nm ± 136

inhibitory activity as aggregated particles were removed from the supernatant leaving solely monomers behind. DLS analysis also revealed the development of colloidal aggregates since the size of inhibitor particles increased from 63 nm ± 6–236 nm ± 80 (approximately 4-fold growth) within 20 min, whereas the protein size remained unchanged (130 nm ± 20) during this period.

For derivative **5**, Triton X-100 considerably increased the inhibitory potency instead of attenuating it, moreover, the degree of inhibition remained unchanged in the presence of higher PPA concentrations. On the other hand, the total loss of inhibition due to centrifugation indicated that an aggregate of the inhibitor molecules was likely to represent the active form towards PPA. Furthermore, based on the obtained results, we could propose that a rapid aggregation of inhibitor molecules took place which can be proved by the fact that after incubating the enzyme and inhibitor for 5 min, the IC_{50} decreased by 38%, and there was no further improvement in the inhibition after 30 min either. Therefore, 5 min appear to be sufficient for the formation of the large-sized aggregate, which was able to adsorb most of the protein molecules. In addition, this assumption was confirmed by DLS experiments: we detected inhibitor particles of 506 nm ± 104 already in the very first measurement, which was carried out immediately after the sample preparation, and subsequently this size was retained. This reactivity was also shown in the enhanced inhibition under the influence of 0.01% Triton X-100; IC_{50} reduced by 97%, whereas the presence of 0.1% detergent gave rise to a 3.3-fold decrease in the IC_{50} value. Although the detergent might prevent the association of inhibitor molecules, it is also possible that hydrophobic interactions are established between the inhibitor and the alkylphenyl hydrophobic group of Triton X-100 [52]. Therefore, the more detergent molecules are present, the more detergent-inhibitor complexes will be evolved. These adducts might provide larger surfaces for enzyme molecules to bind to. This effect of detergent was also observed for compounds **6** and **7**. The inhibition percentage produced by derivative **6** increased by 18% in the presence of 0.1% Triton X-100, whereas in the case of analogue **7** the abnormal behaviour completely disappeared, and we obtained well-defined curves in the inhibition assays, with slopes unambiguously determined. The results obtained for derivative **6** showed similarity to those for compound **2**; in fact, for both compounds, the decreased inhibition due to the use of higher enzyme concentration, along with the total loss of inhibitory activity as a result of centrifugation provided an evidence for aggregation, whereas PPA-inhibitor pre-incubation led to decreased inhibitory effect instead of increasing potency. However, DLS measurements revealed that in the case of compound **6**, the aggregation of protein molecules took place instead of the self-association of inhibitor molecules.

When investigating the criteria for the last inhibitor (compound **7**), we were not able to observe a well-defined tendency in the changes of IC_{50} values since we did not obtain an initial value that might be used for a comparison due to the abnormal behaviour in the case of the original inhibition assay. However, we found that 0.01% detergent or 5-min pre-incubation with the enzyme, or the use of 6-fold higher enzyme concentration were required so that we could prevent or slow down aggregation allowing us to measure the increasing absorbance values over time. Although the complete loss of inhibitory activity following centrifugation suggests that inhibitor molecules should suffer aggregation, the higher activity observed as a consequence of multiple-fold PPA concentration was indicative of the aggregation of enzyme molecules. DLS experiments proved that both of these assumptions were correct; the size of the inhibitor particles and that of enzyme molecules became approximately 3.5-fold and 6.5-fold larger, respectively, during 5 min.

5. Conclusion

Thiazolidinone-based compounds have been considered as promising drugs for controlling hyperglycaemia in patients suffering from type 2 DM and preventing DM-associated complications either as single-targeted or multi-targeted agents [19]. Recently, *via* a knowledge-based approach, several thiazolidinone derivatives were designed and synthetized, in which the core structure was provided with specific substituents capable of simultaneously interacting with two or more enzymes implicated in DM and its long-term complications [28]. In this study, we extended the investigation on representative (5-arylidene-4-oxo-2-thioxothiazolidin-3-yl)acetic acids (compounds **1**–**7**) by evaluating their inhibitory effects against PPA and α-glucosidase.

Some of the tested compounds were shown to inhibit both of these enzymes at micromolar concentrations. Since most of the tested inhibitors displayed activity against three unrelated enzymes, we decided to evaluate the mechanism of their multiple action in order to discover if aggregation contributes to the inhibition of the target enzymes. Our investigation demonstrated that 4-thiazolidinone derivatives **1**, **3** and **4** are able to act as specific multi-target inhibitors without forming aggregates, whereas the indicators of a likely aggregation-based mechanism of action emerged for analogues **2**, **5**–**7**.

Overall, our present and previous findings corroborate the potential value of 4-thiazolidinone derivatives as bioactive molecules capable of establishing specific interactions with different enzymes. As highlighted above, the evaluation of the possible mode of action of these inhibitors is an important step of their development as potential drugs in order to avoid false positives. However, the behaviour of potential aggregators could change in biological environments, also considering that many well-known marketed drugs displayed aggregator properties *in vitro* [38], and this could be further evaluated.

Out of the tested 4-thiazolidinone derivatives, compounds **2**, **5**–**7** appeared to be slightly aggregating compounds. It might be reasonable to expect that they do not form aggregates at concentrations lower than the high micromolar amounts used in this work towards PPA enzyme; in particular, their AR inhibitory effects, which are displayed at much lower submicromolar concentration, could be due to a specific inhibition. On the other hand, compounds **1**, **3**, and **4** exhibited a promising profile since they were shown to act without producing aggregates even at high micromolar concentrations. They are potent inhibitors of human AR, with moderate capability also for inhibiting PPA, α-glucosidase and PTP1B. Moreover, the biological properties of compounds **1**, **3**, and **4** also includes anti-inflammatory activity, which we had previously assessed in human keratinocyte cultures exposed to IFN-γ and histamine [22]. The activity profile of derivative **4** appears to be the most interesting as it is more active against both PPA and α-glucosidase and showed more marked anti-inflammatory potency compared with analogues **1** and **3**. Based on these activities, compounds **1**, **3**, and **4** could be further developed as potential agents for the treatment of DM and its complications. Further studies will include the design of suitable structural modifications of these compounds in order to balance their activities, in particular to improve inhibition of PPA, α-glucosidase and PTP1B as well as to better delineate SARs for this class of MTL.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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