



The antidiabetic drug lobeglitazone has the potential to inhibit PTP1B activity

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ABSTRACT

Protein tyrosine phosphatase 1B (PTP1B) is considered a potential therapeutic target for the treatment of type 2 diabetes mellitus (T2DM), since this enzyme plays a significant role to down-regulate insulin and leptin signalling and its over expression has been implicated in the development of insulin resistance, T2DM and obesity. Some thiazolidinediones (TZD) derivatives have been reported as promising PTP1B inhibitors with anti-hyperglycemic effects. Recently, lobeglitazone, a new TZD, was described as an antidiabetic drug that targets the PPAR- γ (peroxisome γ proliferator-activated receptor) pathway, but no information on its effects on PTP1B have been reported to date. We investigated the effects of lobeglitazone on PTP1B activity *in vitro*. Surprisingly, lobeglitazone led to moderate inhibition on PTP1B (IC_{50} 42.8 ± 3.8 μ M) activity and to a non-competitive reversible mechanism of action. As lobeglitazone inhibits PTP1B activity *in vitro*, we speculate that it could also target PTP1B signalling pathway *in vivo* and thus contribute to potentiate its antidiabetic effects.

1. Introduction

Protein tyrosine phosphatases (PTPs) as potential therapeutic targets have been an important topic in biomedical science for the last two decades, because a large number of PTPs dysfunctions are associated with several human diseases, such as cancer, diabetes, autoimmune and neurological diseases [1–6]. Several years of study revealed that protein tyrosine phosphatase 1B (PTP1B), which is a negative regulator of insulin and leptin signalling pathway [5–14], plays an important role in the development of many diseases, including diabetes and obesity. Knockout studies in mice models show that animals that do not express PTP1B present insulin hypersensitivity and low blood glucose levels [14,15]. Furthermore, the gene encoding PTP1B is located in chromosome 20q13, a genomic region associated with type 2 diabetes mellitus (T2DM) [16]. Also, PTP1B overexpression contributes to the development of insulin resistance, T2DM and obesity [17]. Insulin exerts its effects when it binds to insulin receptor protein tyrosine kinase, which initiates signalling pathways, particularly those that induce phosphorylation. Diabetes is a disease, related to insulin resistance, in which signalling responses to hormones are attenuated [13]. According to Krishnan et al. an ideal therapeutic target would be one that functions

as a negative regulator of both signalling pathways, where their inhibition would restore a normal hormonal response [18].

PTP1B is one such target that counteracts tyrosine phosphorylation events by promoting insulin signalling and simultaneously interrupts glycaemic control. Hence, PTP1B inhibition has been validated as a potential therapeutic strategy to enhance insulin sensitivity, and inhibitors of this enzyme are being suggested as potent antidiabetic drugs [19]. Numerous PTP1B inhibitors have been reported so far, including natural products, synthetic heterocyclic or hybrid compounds, but only a few have reached clinical trials. However, none of them have reached the market yet [20].

There are currently several treatment options available to control T2DM, in particular antidiabetic agents that act as hypoglycemic drugs, such as thiazolidinediones (TZDs), which are synthetic peroxisome γ proliferator-activated receptor (PPAR- γ) agonists [21–23]. Activation of PPAR- γ increases glucose uptake and use in peripheral organs, in addition to fatty acid storage in adipocytes, insulin signalling and decreases liver gluconeogenesis, thereby improving insulin sensitivity [21,24,25]. TZDs also affect a range of activities, including cell proliferation, apoptosis, inflammation, and responses to oxidative stress [26,27]. Although effective, TZDs, such as rosiglitazone and

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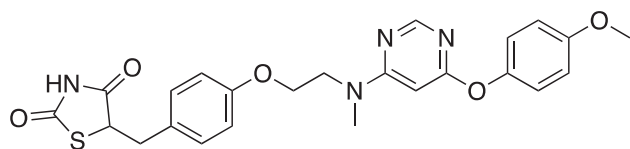


Fig. 1. Structure of Lobeglitazone.

pioglitazone, are associated with weight gain, increased risk of heart failure, and bladder cancer among others [28–30].

Recently, a new PPAR- γ agonist was developed with a TZD moiety and substituted pyrimidines, lobeglitazone (Duvie®; Fig. 1) is structurally similar to rosiglitazone and pioglitazone, two well-known TZD drugs [31–36]. Phase III trials have shown that glycosylated haemoglobin levels were decreased following lobeglitazone monotherapy and as a complement to metformin therapy. This drug was approved by the Ministry of Food and Pharmaceutical Safety (Korea) in 2013 and has since been used as a daily treatment of T2DM [37,38]. As PTP1B inhibition improves the sensitivity to insulin and some TZD analogues have been reported as PTP1B inhibitors [39–45], we sought to determine the effect of lobeglitazone on PTP1B activity *in vitro*. Lobeglitazone is used as an antidiabetic drug that targets PPAR- γ pathway, but no information regarding its effects on PTP1B is reported.

2. Results and discussion

Initially, *in vitro* inhibitory activity of lobeglitazone against PTP1B was assessed spectrophotometrically at 40 μM using *p*NPP as substrate. Lobeglitazone reduced $41.5 \pm 2.7\%$ of enzymatic activity with an IC_{50} value of $42.8 \pm 3.8 \mu\text{M}$, which means it is a moderate inhibitor of PTP1B.

Several TZD derivatives have been reported as PTP1B inhibitors with antihyperglycemic and antiobesity effects [39–45]. Liu et al. discovered and optimized a series of new PTP1B inhibitors that contain TZD-substituted biphenyl scaffold and further evaluated the inhibitory effects of these compounds *in vitro* and *in vivo* [44]. Among them, compound (Z)-5-((4'-(benzyloxy)biphenyl-4-yl)methylene)-2-thioxothiazolidin-4-one (7Fb), derived from 4 oxothiazolidine-2-thione, exhibited an IC_{50} value of $0.69 \pm 0.07 \mu\text{M}$ for PTP1B. The compound significantly decreased postprandial and fasting glucose levels, and blood glucose levels decreased more rapidly than in mice treated with metformin, which demonstrates its potency as an antidiabetic agent. A number of new 5-benzene lidene-2,4-thiazolidinedione derivatives have been designed and synthesized by Wang et al. based on their previous studies [45]. Compound (Z)-5-(5-bromo-4-((3-methylbut-2-en-1-yl)oxy)-2-methoxybenzylidene)-3-isopropylthiazolidine-2,4-dione (7e) exhibited potent competitive inhibition of PTP1B, with an IC_{50} value of $4.6 \mu\text{M}$. Although lobeglitazone seems to have moderate activity against PTP1B, comparing its potency with other inhibitor described for this enzyme, we cannot exclude that it might have some inhibitory activity of PTP1B *in vivo*, since Mahapatra et al. reported two TZD derivatives with IC_{50} values of $\approx 7.5 \mu\text{M}$ that significantly reduced the blood sugar level *in vivo* relative to standard drug pioglitazone.

Based on the IC_{50} value, we investigated the mode of inhibition of lobeglitazone by evaluating the PTP1B kinetic parameters. Kinetic analysis revealed that lobeglitazone is a non-competitive inhibitor ($\alpha = 1$) of PTP1B (Fig. 2), with a K_i value of $61.0 \pm 9.0 \mu\text{M}$.

The finding that lobeglitazone has a non-competitive mechanism of inhibition may represent an important feature because this type of inhibition has demonstrated potential to lead to selectivity and potency. Recently, research has advanced towards the development allosteric inhibitors of PTPs. Trodusquemine (MSI-1436) a reversible non-competitive allosteric inhibitor of PTP1B attenuates its ability to promote HER2-dependent tumorigenesis *in vivo* and reached phase I clinical trials for breast cancer and phase 2 clinical trials for T2DM [47]. This promising PTP1B allosteric inhibitor has boosted research in the field of

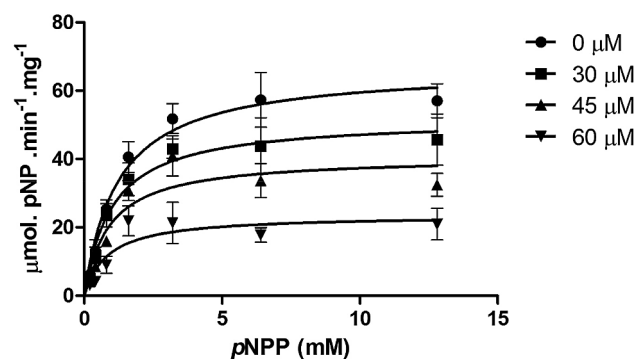


Fig. 2. Michaelis–Menten chart that represents the inhibition mechanism for lobeglitazone against PTP1B.

PTP-based drug discovery [18]. Thus highlighting the importance of the search for non-competitive inhibitors of these enzymes, mainly because their active sites are well conserved among the family [48,49].

Besides, different types of inhibitions are described for TZD inhibitors of PTP1B, which includes non-competitive mechanisms, but all the reports indicate reversible inhibition [40–42,46]. Thus, to determine whether the inhibition was caused by the formation of a reversible or irreversible enzyme-inhibitor complex, we investigated the recovery of the phosphatase activity by a pre-incubation and dilution assay [50]. The results demonstrated that the recovery of activity for lobeglitazone was $93.0 \pm 5.0\%$, which means it behaves as a reversible inhibitor of PTP1B (Fig. 3). This also potentially excludes the formation of colloidal aggregates by lobeglitazone that could lead to local enzyme denaturation and false positive readouts [59].

To corroborate the results of the kinetic analysis, we conducted a limited proteolysis assay to understand the availability of the active site of the enzyme for proteolysis. First, we analyzed the PTP1B peptide mass fingerprint (PMF) in the presence and absence of lobeglitazone. Approximately 86.3% of the PTP1B sequence was covered in the PMF analysis in the presence and 76.3% in the absence of lobeglitazone. By comparing the PMF spectra, we observed that peptides of the PTP1B active site were not protected by the presence of the compound, the peaks 2175 and 2430 m/z (Fig. S1 and S2 Supplementary material) remain the same, which confirms that lobeglitazone is a non-competitive inhibitor, however further studies are needed to determine the binding site of this inhibitor on the enzyme.

Our results reveal that lobeglitazone has an inhibitory activity of PTP1B *in vitro*, thus despite its weaker PTP1B activity, this partial inhibition could contribute positively to the effects observed with its primary target PPAR- γ . Some studies have shown the effect of insulin sensitization due to the combination of activating PPAR- γ and inhibiting PTP1B activity [42,43,51,52]. Kubo et al. compare the pharmacological profile of two PPAR- γ agonists, the KY201 (EC_{50} : $287 \pm 88 \text{ nM}$) and rosiglitazone (EC_{50} : $148 \pm 39 \text{ nM}$) in ovariectomized rats [51]. KY201, but not rosiglitazone, inhibited PTP1B

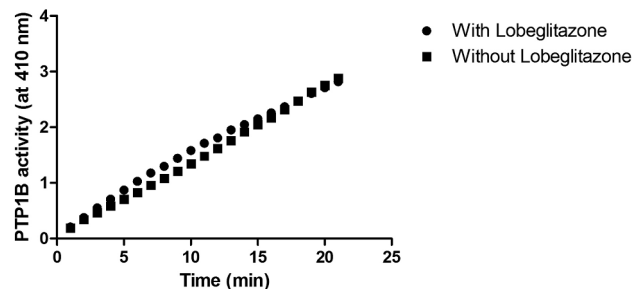


Fig. 3. Reversibility of PTP1B inhibition by lobeglitazone. Representative graph of three independent experiments.

(IC₅₀: 3.7 ± 1 μM) and increased insulin receptor phosphorylation in HepG2 cells. These results suggest that the effects of KY201 are similar to those of rosiglitazone, but with less adverse effects due to the combination of partial PPAR-γ activation and PTP1B inhibition. Bhattarai et al. also reported the efficacy of new TZD derivatives with dual action as PTP1B inhibitors and PPAR-γ activators, to suppress weight gain and improve blood parameters [43].

Current studies continue to demonstrate the importance of PTP1B inhibition as a potential target for the treatment of T2DM [6,53–55]. Ottanà et al. designed and evaluated 3-aryl-5-arylidene-2-thioxo-4-imidazolidinones as a new class of non-carboxylated PTP1B inhibitors that act as reversible non-competitive inhibitors and as potent insulin-sensitizing agents [53]. Their results demonstrate that these non-competitive PTP1B inhibitors can be considered promising compounds to improve the druggability of the target enzyme. Recently, Liu et al. designed and synthesized 17 new 4-thiazolinone derivatives as new PTP1B inhibitors for the treatment of T2DM [6].

3. Conclusions

In conclusion, we showed that the potent PPAR-γ agonist lobeglitazone is a PTP1B inhibitor *in vitro*, with an IC₅₀ and K_i values of 42.8 ± 3.8 μM and 61.0 ± 9.0 μM, respectively. Furthermore, lobeglitazone presented a reversible non-competitive-type inhibition against PTP1B. This finding suggests that lobeglitazone might have potential to inhibit PTP1B *in vivo*, as has been shown for other PPAR-γ agonists. Thus, this partial inhibition could have a positive off-target effect on lobeglitazone treatment, complementing PPAR-γ activation. Consequently, these results may stimulate further understanding of current treatments for T2DM with lobeglitazone, lead to the development of new strategies with multiple mechanisms of action and lobeglitazone could be a lead compound for the development of potent PTP1B inhibitor drugs.

4. Materials and methods

4.1. Expression and purification of recombinant human PTP1B

To perform PTP1B expression and purification, the protocol previously described by Brandão et al. was used [56]. After purification, the protein was concentrated by ultrafiltration with a 30 kDa pore membrane (Amicon Ultra-15 Millipore). The final protein concentration was determined by Bradford assay [57].

4.2. Biochemical evaluation

PTP1B phosphatase activity was tested by using pNPP as substrate. Firstly, the recombinant protein (38 nM) was incubated at 37 °C for 10 min in imidazole buffer (20 mM), pH 7.0, using 40 μM of lobeglitazone (stock dissolved in DMSO) at 4% DMSO. The reaction was initiated by adding pNPP (5 mM), the quantity of *p*-nitrophenol produced was monitored spectrophotometrically at 410 nm for 10 min. Analysis of the enzymatic activity was carried out in 96-well plates and the absorbance was measured on an ELISA plate spectrophotometer (Multiskan FC). The enzyme with DMSO (4%) was considered as positive control and the absence of enzyme was the negative control. The enzymatic activity was expressed in % of phosphatase residual activity relative to wells without inhibitor. All assays were done in triplicate in at least three independent experiments. The IC₅₀ values were determined by increasing concentrations of inhibitor (1 to 100 μM) versus % of inhibition, in triplicate in at least three independent experiments. The experimental data was analysed with GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA) and the IC₅₀ values determined by linear regression.

4.3. Kinetic measurements

To determine the kinetic parameters and the mechanism of inhibition, three concentrations of the compound, by using the IC₅₀ value as reference, and seven pNPP concentrations were used (0.2, 0.4, 0.8, 1.6, 3.2, 6.4 and 12.8 mM). V_{max} and K_{mapp} values were determined for each compound concentration by nonlinear adjustment of the Michaelis–Menten equation data. Kinetic parameters and inhibition constant (K_i) were determined by nonlinear regression of the data according to the specific equation for each inhibition mode. All data were generated and plotted by using the GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA). All assays were done in triplicate in at least three independent experiments.

4.4. Enzyme activity reversibility assay

Recovery of enzymatic activity was determined after a large and rapid dilution of the enzyme inhibitor complex [58]. Control (enzyme in 10% DMSO) and inhibition reactions (lobeglitazone at 10 fold IC₅₀ value) were previously incubated in imidazole (20 mM, pH 7.0) for 30 min at 37 °C. Afterwards, 2 μL of this mixture was diluted 100-fold in a solution with imidazole (20 mM) and pNPP (10 mM). The amount of pNP produced was measured at 410 nm for 20 min (with readings every minute) at 37 °C. All assays were performed in triplicate.

4.5. Limited proteolysis of enzyme-inhibitor complex

A proteolysis assay of the enzyme inhibitor complex was performed by using trypsin protease (Trypsin Gold, Mass Spectrometry Grade, Promega). In this analysis, PTP1B at a final concentration of 5 μM was incubated with lobeglitazone at a final concentration of 100 μM at 4% DMSO or with 4% DMSO in a 25 mM ammonium bicarbonate solution pH 7.5 for 10 min at 37 °C. After this period, protease was added (10 μg/ml with a protease-protein ratio of 1:50). The digestion reaction was incubated at 37 °C for 1 h. Then, 1 μL aliquots of the reaction was transferred to a microtube that contained 3 μL of the saturated solution of the alpha-cyano-4-hydroxycinnamic acid matrix (5 mg/50% acetonitrile, 0.1% trifluoroacetic acid). Then 1 μL of this mixture was applied directly to the MALDI-TOF/TOF Autoflex III Smartbeam (Bruker Daltonics) spectrometer plate and crystallized at room temperature. After crystallization of the sample, mass spectrometry analysis was performed in positive mode. The spectra were analysed by using the Flex Analysis 3.3 program. The PMF profile obtained by MS was identified by comparison with the theoretical digestion peptide list obtained through the Sequence Editor program (Bruker Daltonics). All assays were performed in triplicate [58].

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 material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bioorg.2020.103927>.

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