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RESEARCH ARTICLE

Influence of analgesic active 3-[4-(3-trifluoromethyl-phenyl)-piperazin-1-yl]-dihydrofuran-2-one on the antioxidant status, glucose utilization and lipid accumulation in some *in vitro* and *ex vivo* assays

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Abstract

Purpose: Earlier we demonstrated that 3-[4-(3-trifluoromethyl-phenyl)-piperazin-1-yl]-dihydrofuran-2-one (LPP1) elevates nociceptive thresholds in the mouse model of diabetic neuropathic pain. Since drug-induced impairments of glucose and lipid metabolism and the oxidative stress might diminish benefits from analgesia achieved by analgesic drugs used in diabetic neuropathy, the effect of LPP1 on glucose utilization, lipid accumulation and its antioxidant and cytotoxic potential were assessed in some *in vitro* and *ex vivo* tests.
Methods: Total antioxidant capacity was evaluated spectrophotometrically using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical method, whereas the activities of glutathione (GSH) peroxidase and reductase were measured using methods based on the oxidation of NADPH to NADP. The spectrophotometric method for the evaluation of GSH level in mouse brain tissue homogenates involved the oxidation of GSH by the sulfhydryl reagent 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) to form a yellow derivative, 5′-thio-2-nitrobenzoic acid (TNB), measurable at 412 nm. Cytotoxicity and glucose utilization were measured in hepatoma HepG2 cells and in 3T3-L1 adipocytes. Lipid accumulation was measured in 3T3-L1 cell lines.
Results: LPP1 had dose-dependent antioxidant properties in DPPH radical assay (14–22% versus control; $p < 0.001$). Its single administration caused an increase in GSH concentration in brain tissue homogenates of mice by 34% (versus control group; $p < 0.05$). LPP1 was not cytotoxic and it did not increase glucose utilization or lipid accumulation in cell cultures.
Conclusions: Previously demonstrated antinociceptive properties of LPP1 are accompanied by a lack of cytotoxicity. LPP1 does not impair glucose or lipid metabolism and is an antioxidant. All these properties might be advantageous for its use in diabetic neuropathy.

Keywords

Cytotoxicity, dihydrofuran-2-one, glucose utilization, glutathione, lipid accumulation

History

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Introduction

In the recent years diabetes mellitus (DM) has become one of the most prevalent metabolic diseases in the world with approximately 180 million of people suffering from the disease itself or its complications. The pathogenesis of DM is very complex and not fully understood. It is characterized not only by the elevated blood glucose level but also by the other metabolic impairments, including hypertriglyceridemia, insulin resistance and oxidative stress.

Type 2 DM is a metabolic syndrome in which glucotoxicity and lipotoxicity play a pivotal role. It is thought that both these phenomena can exert their damaging or toxic effects on pancreatic β-cells (Poitout & Robertson, 2008). At the advanced stage of type 2 DM metabolic disorders: elevated glucose and triglyceride levels together with insulin resistance often coincide with the development of peripheral neuropathies which are characterized by severe, paroxysmal or chronic pain episodes called painful diabetic neuropathy (PDN).

PDN, a significant cause of diabetes-related morbidity and mortality, results from the direct toxic effect of glucose on nerve cells (Pluijms et al., 2011) but many additional risk factors can be distinguished, including age, diabetes duration, lipotoxicity, genetic factors, inflammation and oxidative stress (Chong & Hester, 2007; Vinik, 2005). The modification of the underlying disease (''disease-modifying treatment'' to reduce its severity: tight glycemic control, the maintenance of body weight and normal lipid levels, the use of aldose reductase inhibitors, protein kinase C beta

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Abbreviations

DM: diabetes mellitus
DPPH: 2,2-diphenyl-1-picrylhydrazyl radical
DTNB: 5,5′-dithio-bis-(2-nitrobenzoic acid)
GSH: glutathione
LD_{50}: median lethal dose
PDN: painful diabetic neuropathy

inhibitors, antioxidants, e.g. alpha lipoic acid and transketolase activators) and control of pain symptoms with the use of analgesic drugs, mainly analgesic adjuvants, are two mainstreams of the treatment of PDN (Bril, 2012; Chong & Hester, 2007; Pluijms et al., 2011; Satoh et al., 2011; Vinik, 2005, Ziegler, 2008; Zilliox & Russell, 2011). Analgesic drugs used to relieve pain in diabetic patients should therefore exert their therapeutic effect without aggravating glucose tolerance or inducing lipid accumulation. In addition, apart from this highly desirable metabolic neutrality they should be devoid of cytotoxic properties and not interact with other drugs used in diabetic patients, in particular with oral hypoglycemic agents.

In our previous studies we have demonstrated that 3-[4-(3-trifluoromethyl-phenyl)-piperazin-1-yl]-dihydrofuran-2-one (LPP1) has significant antinociceptive properties in mouse models of acute pain evoked by thermal and chemical stimuli (Salat et al., 2009, 2012, 2013a) and these results directed further research on this analgesic active compound and its safety profile. Recently, we have shown that LPP1 is a very efficacious antiallodynic and antihyperalgesic agent that reduces symptoms of PDN in the mouse model of streptozotocin-induced diabetic neuropathy (Salat et al., 2013a). We have also demonstrated that this compound potentiates the antiallodynic effect of pregabalin in diabetic mice (Salat & Salat, 2013) and has antioxidant properties in some *in vitro* tests (Salat et al., 2012, 2013a). In the present study, we further assess the antioxidant capacity of LPP1 and its effect on selected parameters of the enzymatic and non-enzymatic antioxidant defense. The effects of LPP1 on glucose utilization and lipid accumulation, as well as its possible adverse cytotoxic influence on cells have not yet been investigated. Such knowledge is pivotal for further evaluation of this novel analgesic active compound. The influence of LPP1 on glucose utilization and lipid accumulation might have an impact on the progress of DM and the development of PDN. The diabetes itself worsens the conditions of neural tissue, so drugs used in the treatment of neuropathic pain syndromes should not have their own cytotoxic effect on cells (Arezzo et al., 2008). Hence, a possible cytotoxic effect of this compound is also assessed.

Materials and methods

Animals and housing conditions

For *ex vivo* assays brains of adult male Albino Swiss (CD-1) mice weighing 18–22 g were used. The animals were provided by the Animal Breeding Farm of the Jagiellonian University in Cracow. They were kept in groups of 15 mice in cages at a room temperature of $22 \pm 2\,^{\circ}C$, under light/dark (12:12) cycle and had free access to food and water. To evaluate the influence of LPP1 on glutathione (GSH) peroxidase, GSH reductase and GSH levels in mouse brain tissue homogenates, this compound was suspended in a 0.5% methylcellulose solution (Loba Chemie, Freiberg, Germany) and was administered at a dose of 30 mg/kg by the intraperitoneal (i.p.) route. This dose was chosen for *in vivo* and further *ex vivo* tests based on its previously established high efficacy in pain tests. LPP1 was administered either as a single dose or as a repeated-dose protocol (10-day administration). Control animals were given an appropriate amount of vehicle (0.5% methylcellulose suspension; i.p.). Then the mice were killed and their brains were isolated and prepared as described below. All these procedures were approved by the Local Ethics Committee of the Jagiellonian University in Cracow (ZI/595/2011).

Chemicals used in pharmacological tests

LPP1 was synthesized at the Laboratory of Physicochemical Drug Analysis, Chair of Pharmaceutical Chemistry, Faculty of Pharmacy, Jagiellonian University in Cracow. Other drugs used throughout the assays – rosiglitazone and terfenadine were provided by Sigma Aldrich (Poznań, Poland) and Tocris Bioscience (Wiesenbaden-Nordenstadt, Germany), respectively. Quercetin and α-tocopherol (trolox) were supplied by Polskie Odczynniki Chemiczne (Gliwice, Poland).

In order to evaluate cytotoxic effects and the influence of LPP1 on glucose utilization and lipid accumulation, stock solution (1 mM) of LPP1 was prepared in PBS (phosphate buffer, Polskie Odczynniki Chemiczne, Poland). Serial dilutions of LPP1 were prepared in PBS and added to cell cultures to give final concentrations of 1, 10, 50 and 100 μM. Concentration regimens of LPP1 and the reference drugs for the *in vitro* assays were chosen based on the literature data and our previous studies (Salat et al., 2012, 2013b). The samples were tested in triplicates in two independent experiments. All measurements were performed using a microplate reader POLARstar Omega (BMG Labtech, Cary, NC).

Evaluation of the antioxidant capacity using 2,2-diphenyl-1-picrylhydrazyl radical method

The antioxidant capacity of LPP1 (1, 10, 50 and 100 μM) was assessed spectrophotometrically using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical method as recently described by Salat et al. (2013b). Briefly, in this assay the absorbance decreases when DPPH radical is reduced by antioxidants. The absorbance was measured at a wavelength of 515 nm after a 15-min incubation period with 0.2 mM DPPH. Quercetin and trolox were chosen as positive controls in this test as they both possess strong antioxidant capacities (Bai et al., 2013; Zhang et al., 2011).

Evaluation of the antioxidant capacity in mouse brain tissue homogenates

For the evaluation of antioxidant capacity of LPP1, the whole brain tissues of mice that received this compound as a single dose or a repeated-dose protocol were homogenized in a 0.05 M potassium phosphate buffer (pH 7.0) to obtain 20%

homogenates. The homogenates were centrifuged ($800 \times g$, 20 min, 4 °C) and the supernatants were used in all further assays. Protein concentrations were measured by means of a biochemical analyzer MaxMat PL (Maxmat, Montpellier, France) using ready-made reagents and applications from Allmed (Cracow, Poland).

Influence on GSH peroxidase activity

The activity of GSH peroxidase was measured using a modification of the method described by Paglia & Valentine (1967). The reaction mixture consisted of 40 µl of phosphate buffer (0.05 mol/l, pH 7.0) containing 0.005 mol/l of EDTA, 100 µl of NADPH solution (0.0084 mol/l), 25 µl of glutathione reductase (100 U/mg protein/ml), 100 µl of reduced glutathione (0.015 mol/l), 25 µl of sodium azide (1.125 mol/l) and 25 µl of homogenate. The enzymatic reaction was initiated by an addition of 10 µl of hydrogen peroxide (0.022 mol/l). The decrease in absorbance was measured at 340 nm for 3 min on the biochemical analyzer MaxMat PL. A blank with all ingredients except for homogenate sample was also monitored. The activity of GSH peroxidase in the sample was calculated by the use of the NADPH extinction coefficient at 340 nm, 6.22×103 $mol^{-1}cm^{-1}$ (this value has been adjusted for the path length of the solution in the well – 0.7 cm). The final results were expressed as units per gram of protein.

Influence on GSH reductase activity

The activity of GSH reductase was assayed according to the method of Goldberg & Spooner (1983). This method is based on the oxidation of NADPH to NADP during the reduction of oxidized glutathione. The assay mixture consisted of 250 µl of oxidized glutathione (0.0022 mol/l), 50 µl of NADPH (0.00017 mol/l) and 12 µl of the homogenate. All reagents were prepared in phosphate buffer (0.25 mol/l, pH 7.3) containing 0.0005 mol/l K_2EDTA. The decrease in absorbance was measured at 340 nm for 3 min on the biochemical analyzer MaxMat PL. The activity of GSH reductase in the sample was calculated by use of the NADPH extinction coefficient at 340 nm, 6.22×103 $mol^{-1}cm^{-1}$ (this value has been adjusted for the path length of the solution in the well – 0.7 cm). The final results were expressed as units per gram of protein.

Influence on GSH level

GSH content was estimated by the method described by Beutler et al. (1963). This method is based upon the development of stable yellow color when 5,5′-dithiobis-(2-nitrobeznoic acid) (DTNB) is added to sulfhydryl compounds. Firstly, proteins in the homogenate were precipitated with metaphosphoric acid and removed after centrifugation at $1500 \times g$ (10 min, 4 °C). Subsequently, 100 µl of the obtained supernatant, 120 µl of Na_2H_2PO4 (0.3 mol/l) and 8 µl of DTNB solution (1 mmol/l) were mixed and the absorbance was measured after a 40-s incubation period at 412 nm using a biochemical analyzer MaxMat PL. A series of GSH standards were prepared using pure reduced glutathione (10–70 µmol/l) and run to give a standard curve for the determination of GSH level. The GSH concentration was calculated as µM/l.

Cell cultures

3T3-L1 preadipocyte cell line (1×10^4 per well) was cultured in high glucose – Dulbecco's Modified Eagle's Medium (DMEM: 4 mM/l glutamine, 4500 mg/l glucose, 1500 mg/l sodium bicarbonate) (ATCC, Teddington, UK) with 10% calf serum (ATCC, Teddington, UK), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Gibco, Life Technologies, Grand Island, NY) in a 96-well plates until they were 100% confluent. The confluent cells were then stimulated with DMEM supplemented with 10% calf bovine serum (FCS, ATCC, Teddington, UK) and a 0.5 mM of 3-isobutyl-1-methylxanthine (IBMX, Sigma Aldrich, Poznań, Poland), 1 µM dexamethasone (Sigma Aldrich, Poznań, Poland) for 48 h. The culture medium was replaced with DMEM supplemented only with 10 µg/ml human recombinant insulin and 10% CBS. Cells with LPP1 were incubated for 24 h at 37 °C with 5% CO_2 in sterile conditions.

Hepatocyte HepG2 cell line (2×10^4 per well) was cultured in Eagle's Minimum Essential Medium (EMEM: 1 mM sodium pyruvate, 2 mM/l glutamine, 1500 mg/l sodium bicarbonate), (ATCC, Teddington, UK) with 10% fetal serum (ATCC, Teddington, UK), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Gibco, Life Technologies, Grand Island, NY) in a 96-well plates. Cells with LPP1 were incubated for 24 h at 37 °C with 5% CO_2 in sterile conditions.

Evaluation of cytotoxicity

Cell survival in samples was measured using Prestoblue™ Cell Viability reagent (Life Technologies, Grand Island, NY) according to the manufacturer's protocol. Fluorescence intensity was measured at a wavelength excitation of 530 nm and emission of 580 nm, after a 20-min incubation period at 37 °C. The results are expressed as percentage cell viability in comparison to the control sample. Terfenadine was used as a positive control.

Influence on glucose utilization

The Amplex Red Glucose Kit (Life Technologies, Grand Island, NY) was used to measure glucose utilization by two cell lines (3T3-L1 and HepG2) after incubation with LPP1. Fluorescence intensity was measured at a wavelength excitation filter at 530 nm and an emission filter at 580 nm. The results are expressed as percentage cell glucose utilization in comparison to the control sample. Rosiglitazone was used as a positive control.

Influence on lipid accumulation

The degree of lipid accumulation induced by LPP1 was tested in 3T3-L1 differentiated adipocytes using the AdipoRed test (Lonza, Allendale, NJ) according to the manufacturer's protocol. The cells were incubated with the AdipoRed reagent for 10 min at room temperature. Fluorescence intensity was measured at a wavelength excitation of 485 nm and emission of 535 nm. The results are expressed as percentage lipid accumulation in comparison to the control sample. Rosiglitazone was used as a positive control.

Data analysis

Data analysis of the results was provided by GraphPad Prism Software (v.5, San Diego, CA). The results were statistically

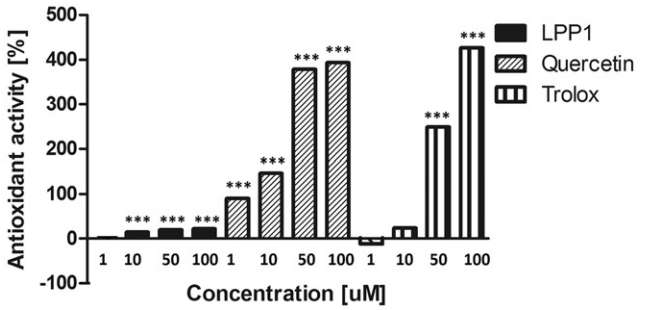

Figure 1. Antioxidant capacity of LPP1 and reference compounds – quercetin and trolox (1–100 μM) in DPPH test. Results are shown as percent of antioxidant capacity of the test compounds compared to control sample ($n = 3$–6). Statistical analysis: one-way analysis of variance (ANOVA), followed by Dunnett's *post hoc* comparison: ***$p < 0.001$.

evaluated using Student's *t*-test or one-way analysis of variance (ANOVA), followed by Tukey's or Dunnett's *post hoc* comparisons to compare the results obtained in drug-treated and vehicle-treated samples. In every case $p < 0.05$ was considered significant.

Results

Antioxidant capacity in DPPH assay

In this assay, LPP1 at 10, 50 and 100 μM demonstrated statistically significant antioxidant properties ($F[4, 19] = 36.32$; $p < 0.0001$) (Figure 1). For these concentrations the antioxidant capacity of LPP1 was 14, 19 and 22%, respectively ($p < 0.001$). Reference compounds: quercetin at each tested concentration and trolox at 50 and 100 μM exerted potent antioxidant effects ranging from 90 to 395% for quercetin ($F[4,19] = 252.0$; $p < 0.0001$), and 250 and 425% for 10 and 100 μM of trolox, respectively ($F[4,19] = 358.9$; $p < 0.0001$).

Influence on GSH peroxidase, GSH reductase and GSH concentration

In these assays LPP1 had no influence on the concentration of both GSH peroxidase ($F[2,26] = 3.119$; NS) or GSH reductase ($F[2,26] = 2.619$; NS) (Figure 2a and b). In contrast, a statistically significant elevation of GSH level was observed after a single dose of LPP1 ($F[2,26] = 5.311$; $p < 0.05$) (Figure 2c).

Cytotoxicity

In HepG2 cell line LPP1 showed no cytotoxic effect ($F[3,20] = 0.0055$; NS) (Figures 3a and 4). In LPP1 samples the percentage of living cells was similar to that of the control sample. A significant cytotoxic effect was observed in the case of a reference drug – terfenadine ($F[2, 27] = 322.8$; $p < 0.0001$) which decreased cell viability by 85% versus control ($p < 0.001$) at the concentration of 50 μM and at 100 μM completely abolished cell viability ($p < 0.001$) (Figures 3a and 4).

The lack of cytotoxicity was confirmed for LPP1 in 3T3-L1 cell line ($F[3,21] = 0.7774$; NS) (Figures 3b and 5). In contrast to LPP1, terfenadine potently decreased cell viability ($F[3, 19] = 266.6$; $p < 0.0001$). At 100 μM it completely abolished cell viability ($p < 0.001$; Figures 3b and 5).

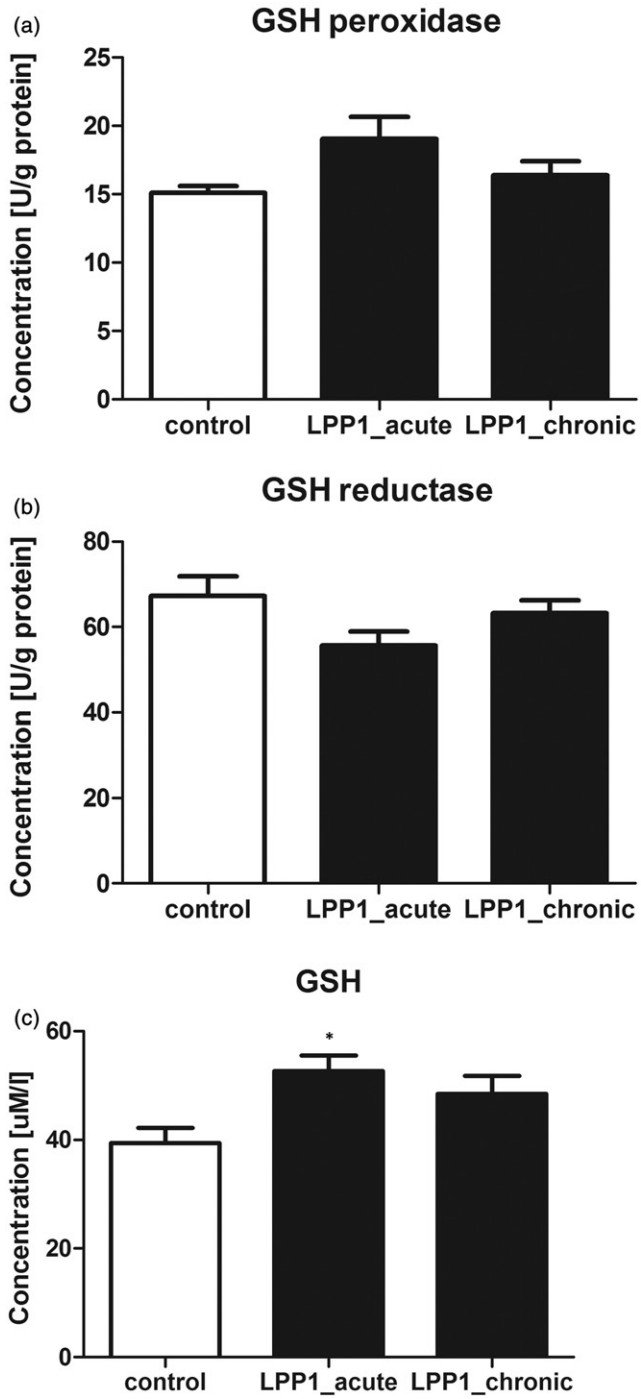

Figure 2. Influence of intraperitoneal LPP1 after a single and a repeated-dose (10-day) administration on GSH peroxidase (a), GSH reductase (b) and GSH (c) levels in mouse brain tissue homogenates ($n = 9$–10). Results are shown as concentrations of GSH peroxidase, GSH reductase or GSH (in [U/g protein] or [μM/l]). Statistical analysis: one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* comparison: *$p < 0.05$.

Influence on glucose utilization

LPP1 did not affect glucose utilization either in HepG2 cells ($F[3,20] = 0.6982$; NS) (Figure 6a) or in 3T3-L1 cells ($F[3, 18] = 1.033$; NS) (Figure 6b). In HepG2 cells rosiglitazone, a positive control, at the concentration of 50 μM

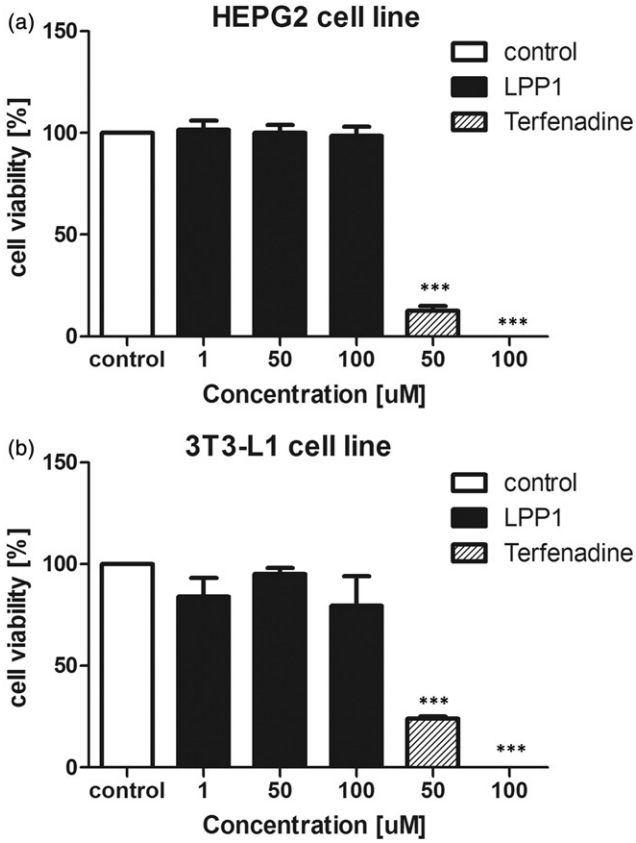

Figure 3. Evaluation of cytotoxicity of LPP1 and terfenadine in HepG2 (a) and 3T3-L1 (b) cell lines. Results are shown as a percentage of cell viability in LPP1, terfenadine and control samples ($n = 6$–12). Statistical analysis: one-way analysis of variance (ANOVA), followed by Dunnett's *post hoc* comparison: ***$p < 0.001$ (versus control sample).

increased glucose utilization by 18.6% versus control ($p < 0.01$). Also in 3T3-L1 cell line rosiglitazone increased glucose utilization ($F[3,11] = 27.23$; $p < 0.0001$). For the concentration of 100 μM this effect was 49.4% ($p < 0.001$) compared to control sample (Figure 6b).

Influence on lipid accumulation

As shown in Figure 7, LPP1 at each tested concentration had no influence on lipid accumulation in 3T3-L1 cell line ($F[3, 30] = 0.5549$; NS), whereas terfenadine at 50 μM and 100 μM significantly decreased lipid accumulation by 28% ($p < 0.05$) and by 66.5% ($p < 0.001$), respectively ($F[3,30] = 19.38$; $p < 0.0001$). Rosiglitazone at 1 μM had no influence on lipid accumulation, whereas at 50 μM and at 100 μM it increased lipid accumulation by 46.1% ($p < 0.01$) and by 62.5% ($p < 0.001$), respectively ($F[3,24] = 16.06$; $p < 0.0001$).

Discussion

Metabolic impairments in DM characterized either by absolute or relative deficiency in the secretion of insulin or insulin action cause chronic hyperglycemia and disturbances of lipid, carbohydrate and protein metabolism (Abou-Seif & Youssef, 2004). Increasing evidence in experimental and clinical studies suggest that metabolic impairments, typically observed in type 2 diabetic patients: insulin resistance, glucolipotoxicity and the oxidative stress (Abou-Seif & Youssef, 2004; Poitout & Robertson, 2008) lead to several fatal complications including β-pancreatic cell dysfunction or their loss, hyperlipidemia, obesity, PDN and the others.

The pathogenesis of PDN is very complex and still not fully understood. It involves *inter alia* poor diabetic control,

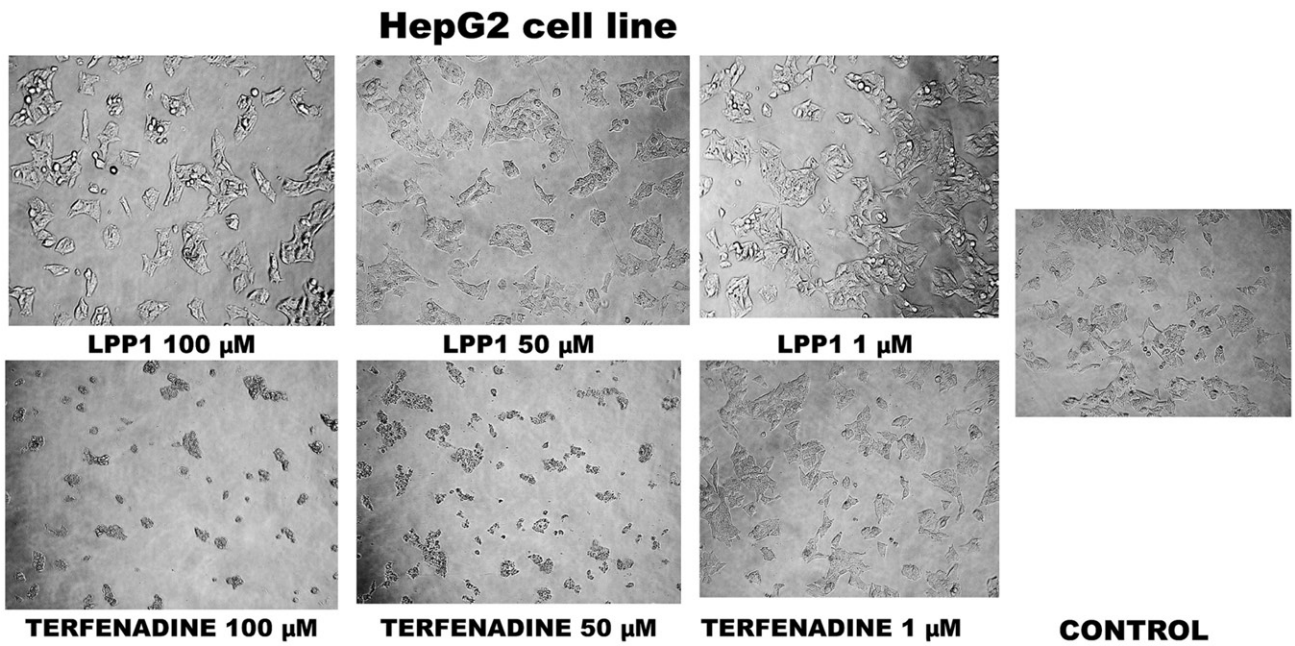

Figure 4. These photos show the microscopic image of HepG2 cell line morphology after the 24-h incubation period with various concentrations (1, 50 and 100 μM) of LPP1 or terfenadine. LPP1 did not alter cell morphology at any of the tested concentrations. Apparent cytotoxic effect on cell morphology was demonstrated when terfenadine was added to cells. Pictures were taken using a microscope Olympus E520 Olympus CKX41 at a magnification of 200×.

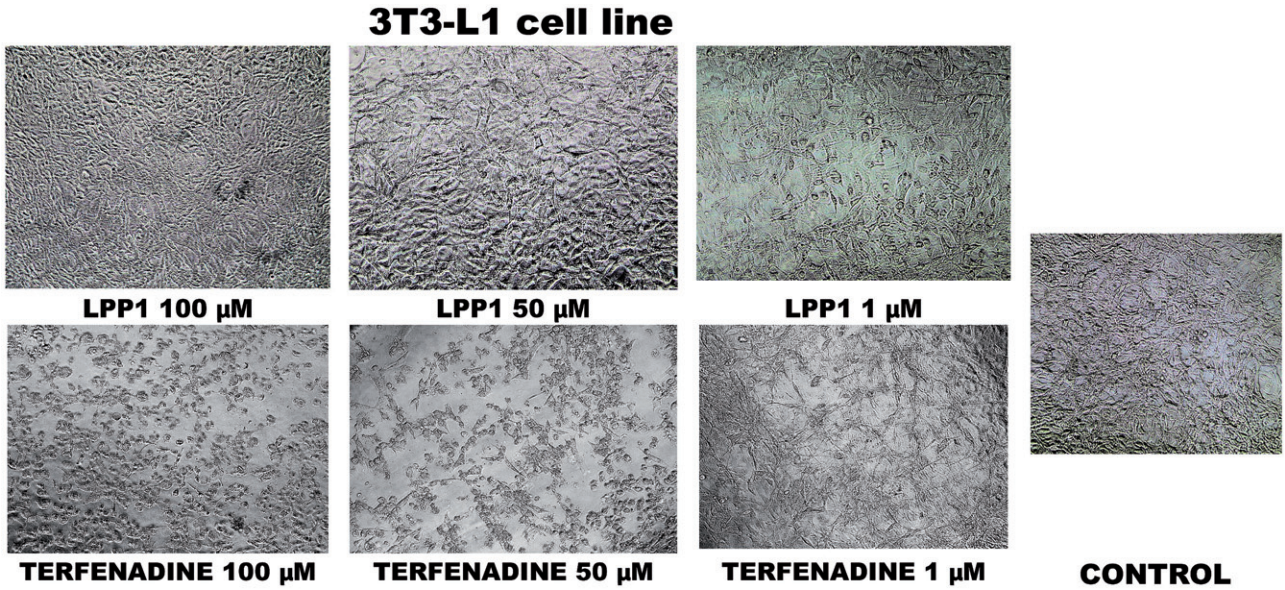

Figure 5. These photos show the microscopic image of 3T3-L1 cell line morphology after the 24-h incubation period with various concentrations (1, 50, 100 µM) of LPP1 or terfenadine. LPP1 did not alter cell morphology at any of the tested concentrations. Apparent cytotoxic effect on cell morphology appeared then terfenadine was added to cell culture. Pictures were taken using a microscope Olympus E520 Olympus CKX41 at a magnification of 200×.

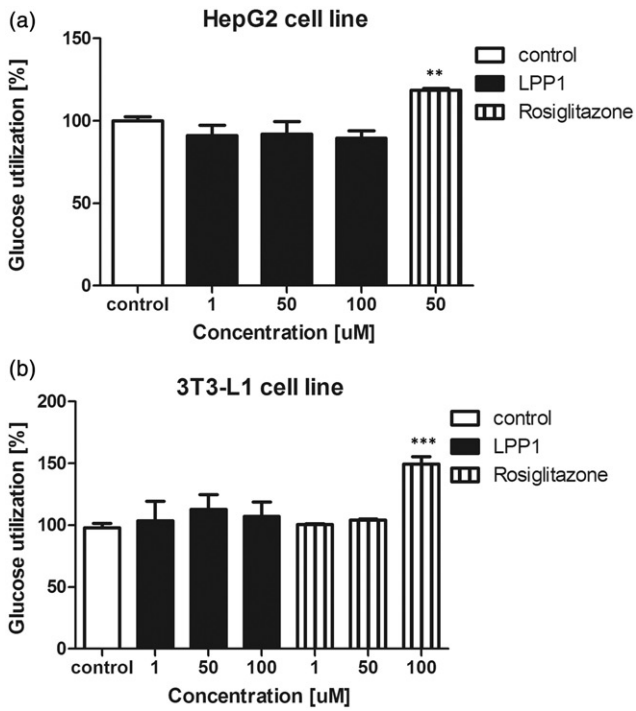

Figure 6. Influence of LPP1 and rosiglitazone on glucose utilization in HepG2 (a) and 3T3-L1 (b) cell lines. Results are expressed as percentage of cell glucose utilization in test samples ($n = 3$–6). Statistical analysis: HepG2 cell line – LPP1: one-way analysis of variance (ANOVA), followed by Dunnett's *post hoc* comparison (results not significant); rosiglitazone – Student's *t*-test: $^{**}p < 0.01$ (versus control); 3T3-L1 cell line: one-way ANOVA, followed by Dunnett's *post hoc* comparison: $^{***}p < 0.001$ (versus control).

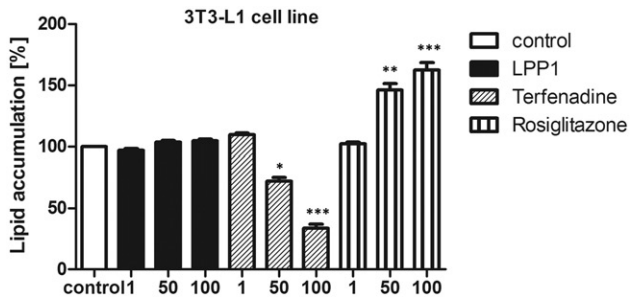

Figure 7. Effect of LPP1, terfenadine and rosiglitazone on lipid accumulation in 3T3-L1 cell line. Results are expressed as percentage of lipid accumulation in test samples ($n = 6$–12). Statistical analysis: one-way analysis of variance (ANOVA), followed by Dunnett's *post hoc* comparison: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (versus control).

elevated triglycerides, oxidative stress and vascular insufficiency which are present during advanced stages of the diabetic process (Chong & Hester, 2007; Salat & Salat, 2013; Tesfaye & Selvarajah, 2012; Zilliox & Russell, 2011). The damage to peripheral nerves, together with the oxidative stress-induced microvascular dysfunction are secondary to chronic hyperglycemia and dyslipidemia and they might also lead to PDN (Pluijms et al., 2011). Accumulating data indicate that glucotoxicity affects nerve cells resulting in PDN (Kuritzky, 2010; Pluijms et al., 2011; Várkonyi & Kempler, 2008). It was also shown that elevated triglycerides are positively correlated with the loss of myelinated fibers, independent of diabetes duration (Tesfaye & Selvarajah, 2012). In view of this, it is clear that drugs used for the treatment of PDN should effectively attenuate pain but they must be metabolically neutral. In particular, they should not impair glucose tolerance or induce lipid accumulation. Also the lack of adverse effects on the nerve structure is highly desirable.

In our earlier research, we demonstrated strong antiallodynic and antihyperalgesic activities of LPP1 in the mouse model of diabetic neuropathic pain induced by streptozotocin. We have also proved that LPP1 did not cause any clear differences in the general structure of the sciatic nerves either in non-diabetic mice or in LPP1-treated diabetic mice

(Salat et al., 2013a). The median lethal dose (LD_{50}) obtained for this compound in the acute toxicity test (747.8 mg/kg; i.p.) was significantly higher as compared to its median effective dose (ED_{50}) values from pain tests (Salat et al., 2009, 2012). Therefore a beneficial therapeutic index for LPP1 is expected. On the other hand, some meaningful neurological adverse effects: impaired motor coordination in the rotarod and chimney tests at doses 100–300 mg/kg and significantly decreased locomotor activity at doses 650–1000 mg/kg were reported previously (Salat et al., 2012).

In the present study, we have shown that this analgesic active compound has no impact on lipid accumulation and glucose utilization and in this context is metabolically neutral. To study these effects of LPP1 we used cell lines which are sensitive to insulin, i.e. differentiated adipocytes and HepG2 cells as a model of organs which are affected by DM and DM-related metabolic disorders. Both hepatocytes and adipocytes play a key role in the regulation of blood glucose level, as well as in the storage of glucose and lipids and the dysfunction of these cells might reflect metabolic disorders during DM. In our research, LPP1 had no influence on glucose utilization and it did not increase lipid accumulation. This metabolic neutrality might be regarded as a favorable property of this compound – also in terms of no risk of drug interactions between LPP1 and antihyperglycemic agents which, as we showed for rosiglitazone, cause a significant enhancement of glucose utilization resulting in the improvement of insulin resistance.

In this study we used rosiglitazone as a positive control. This drug induces adipogenesis in cell culture models and increases glucose uptake in 3T3-L1 adipocytes (Mukherjee et al., 2000) and in hepatocytes (Derlacz et al., 2008). Rosiglitazone, a member of thiazolidinedione drugs is a potent insulin-sensitizing agent which also enhances lipid droplet formation and their accumulation in 3T3-L1 cells. It acts as an agonist of nuclear receptor PPARγ promoting the differentiation of pluripotent stem cells. Adipocytes which are a major site of lipid storage in the body play a crucial role in the maintenance of lipid homeostasis. They regulate energy homeostasis by secreting adipocytokines which affect insulin resistance. Moreover, adipogenesis is insulin-dependent (Li et al., 2007; Pal et al., 2013; Takazawa et al., 2009). An excessive fat accumulation in adipocytes leads to obesity which is one of the risk factors for the development of type 2 DM. Triglyceride accumulation was also proposed as a mechanism underlying β-cell failure in diabetic rats (Poitout & Robertson, 2008). In addition, in isolated islets a prolonged exposure to simultaneously elevated glucose and triglycerides impairs insulin secretion. Recent studies (Poitout & Robertson, 2008) indicate that fatty acids can have a deleterious influence on the function of β-cells. Also, when glucose and fatty acids are elevated, the physiological β-oxidation of fatty acids is blocked and this subsequently adversely affects the function of β-cells. Thus, triglyceride accumulation is regarded as a marker of lipotoxic conditions during DM (Poitout & Robertson, 2008). In addition, lipid accumulation might be the starting point for the process of lipid peroxidation whose products play a key role in the pathogenesis of vascular complications in DM.

In the present study LPP1 did not increase lipid accumulation. In contrast, rosiglitazone enhanced lipid accumulation. This observation is consistent with the results of other authors who also showed similar effects of rosiglitazone on adipose tissue (Takazawa et al., 2009). The second reference drug used in our research – terfenadine, dose-dependently decreased lipid accumulation but this effect could be explained as a result of its undesirable cytotoxic effects on 3T3-L1 cells proven in our study and in the studies of other authors (Hamid et al., 2004) and not its direct influence on lipid accumulation.

Hyperglycemia, as a main cause of DM-related complications is associated with the oxidative stress (Abou-Seif & Youssef, 2004). It was shown that superoxide and nitric oxide are key mediators of glucose-induced oxidative injuries and a marked increase in the whole brain nitrite levels in diabetic animals was observed (Sharma et al., 2006). In diabetic patients, significant deficits of antioxidant protections are observed, such as decreased superoxide dismutase, catalase, glutathione peroxidase and glutathione (Abou-Seif & Youssef, 2004; Kumawat et al., 2013). It is well known that compounds with potent antioxidant capacities (e.g. alpha lipoic acid) reduce the severity of PDN (Chong & Hester, 2007; Kuritzky, 2010). Such agents may aim directly at the pathogenesis of PDN.

To investigate the antioxidant capacity of LPP1 in the DPPH assay, we used quercetin and trolox as positive controls as both these compounds have strong antioxidant properties (Bai et al., 2013; Zhang et al., 2011). We have shown that LPP1 possesses antioxidant properties in DPPH test. Earlier we have also demonstrated that it is antioxidant in the 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical cation scavenging assay (Salat et al., 2012), as well as in the ferric reducing ability of plasma (FRAP) assay (Salat et al., 2013a). Our previous experiments on brain tissue homogenates of mice indicated that a single-dose administration of LPP1 increases the activity of superoxide dismutase without any influence on catalase activity (Salat et al., 2013a). In the present research, LPP1 did not influence the concentrations of GSH peroxidase or GSH reductase but it increased GSH level after a single administration. GSH is an important component of the antioxidant defense of the body and its decrease might reflect a direct reaction between GSH and free radicals generated during hyperglycemia (Abou-Seif & Youssef, 2004; Maritim et al., 2003). However, this advantageous activity of LPP1 was observed only after a single-dose administration and was compensated during the repeated-dose treatment. This might be a limitation of the present study. The effects of repeated-dose application of LPP1, as analgesic drugs are used in neuropathic patients, as well as this compound's influence on glucose utilization and lipid accumulation *in vivo* should be assessed next to gain a further insight into the problem of its safety and potential risk of undesirable adverse effects during a prolonged treatment.

Concluding, the present study aimed at the evaluation of antioxidant capacity and cytotoxicity, as well as the effect on glucose utilization and lipid accumulation of a novel analgesic active dihydrofuran-2-one derivative, the compound LPP1. It has been demonstrated that the *in vitro* test compound

does not increase glucose utilization or lipid accumulation. Moreover, LPP1 has an antioxidant capacity and is devoid of cytotoxic properties. All these features might be very interesting in view of its possible use as an analgesic agent in PDN.

Declaration of interest

The authors declare that they have no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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