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Novel Partial Agonist of PPAR-Gamma for Treatment of Diabetic Neuropathy in Rats

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Abstract

This work describes a novel ligand for peroxisome proliferator-activated receptor gamma (PPARgamma) and its hypoglycemic and analgesic activity in a murine model of diabetes-induced neuropathic pain. The molecular recognition of LASSBio-1772 by a PPARgamma binding domain showed that the compound is a partial agonist. Four weeks after male Wistar rats received a single intravenous injection of streptozotocin (STZ, 60 mg/kg), plasma glucose levels were increased from 92.5 ± 3.7 to 465.0 ± 21.6 mg/dL ($P < 0.01$) and serum insulin was reduced from 66.8 ± 7.8 to 25.5 ± 5.6 pmol/L ($P<0.01$). Diabetic animals were then, treated with either vehicle or LASSBio-1772 (50 mg/kg, i.p.) daily for 7 days. LASSBio-1772 significantly reduced blood glucose levels to 242.0 ± 55.1 mg/dL ($P<0.05$) and increased insulin levels to 58.7 ± 14.8 pmol/L ($P<0.05$), indicating the hypoglycemic activity of this compound. LASSBio-1772 also reduced the elevated triglyceride levels induced by STZ treatment from 74.1 ± 8.6 to 34.0 ± 6.3 mg/dL ($P<0.01$). LASSBio-1772 could reduce serum glucose levels via increased insulin levels and potentially improve insulin sensitivity. Neuropathy was detected after four weeks of STZ-induced diabetes, with reduced thermal heat withdrawal latency from 11.7 ± 0.1 to 7.1 ± 0.2 s ($P<0.01$) and paw withdrawal threshold from 37.5 ± 2.1 to 29.9 ± 1.1 g ($P<0.05$) indicating the establishment of hyperalgesia and allodynia. LASSBio-1772 treatment restored both measures to non-diabetic values (12.2 ± 0.4 s and 34.5 ± 1.5 g). In conclusion, treatment with LASSBio-1772, a partial PPARgamma agonist, decreased hyperglycemia and neuropathic pain induced by diabetes. PPARgamma stimulation by LASSBio-1772 could prevent inflammation and inhibit both peripheral and central sensitization.

Keywords: PPAR-gamma; Diabetic neuropathy; LASSBio-1772; Streptozotocin

Introduction

Diabetes Mellitus (DM) is a prevalent, chronic, non-transmissible disease with high socioeconomic impact. The global occurrence of DM in adults aged 20 years and older in 2000 was estimated to be 171 million, with a projection of 383 million in 2030 [1-3]. Several serious complications can result from poorly controlled DM, including diabetic nephropathy, renal failure, blindness, and atherosclerotic vascular disease [4,5]. In addition, most patients with DM eventually develop neuropathy, with a lifetime risk of lower extremity amputations estimated in some populations to be up to 15%. Hyperalgesia, paresthesias, and allodynia also occurs, with pain evident in 40–50% of those with diabetic neuropathy [6,7]. The substance most commonly used to induce DM in animal model is Streptozotocin (STZ) which can produce progressive death of beta cells. The intravenous dose used to simulates type 1 DM in rats is 65 mg/kg (four to five times less than which is lethal) can cause not only hyperglycemia but also peripheral neuropathy [8].

Peroxisome Proliferator-Activated Receptor (PPAR) is a subfamily of nuclear hormone receptors that regulate gene expression when activated [9]. The PPARγ isoform is expressed mostly in adipose tissue, and selective agonists, including thiazolidinedione derivatives such as rosiglitazone, pioglitazone, and troglitazone, are used clinically to treat DM [10,11]. Troglitazone causes hepatotoxicity but pioglitazone and rosiglitazone do not, suggesting that the toxicity is not related to PPARγ activation. PPARγ activation can regulate gene expression for genes involved in the metabolism of glucose and lipids, insulin sensitivity, cell growth, and differentiation [12,13]. PPARγ is also expressed in immune/inflammatory cells (e.g., monocytes and macrophages), which could contribute to its anti-inflammatory activity [14,15]. PPARγ ligands can reduce the expression of pro-inflammatory genes, decrease TNF-α production, and increase adiponectin expression.

Recently, Zapata-Sudo and coworkers described the compound 3'-[(E)-[2-[(6-methyl-1,3-benzodioxol-5-yl)sulfonyl] hydrazinylidene] methyl]-benzoic acid, or LASSBio-1471, which had favorable theoretical affinity (ΔGbind) for PPARγ in docking studies. Administration of this compound (20 mg/kg, i,p, for 7 days) improved oral glucose tolerance and reduced blood glucose levels in a murine STZ-induced diabetes model. LASSBio-1471 also showed analgesic effects in STZ-induced neuropathy, indicating potential as a novel hypoglycemic agent for pain relief in diabetic neuropathy [16]. To optimize this prototype, LASSBio-1471 was structurally modified using bioisosterism [17] and homologous strategies as shown in Figure 1.

In the present study, we describe the design, synthesis, and evaluation of LASSBio-1772 in a model of diabetes-induced neuropathy. LASSBio-1772 - ((E)-N'-(4-cyanobenzylidene)-N-methylbenzenesulfonyl-hydrazide) was obtained in good yields via the synthetic methodology previously described [16,18].

Figure 1: LASSBio-1772 - ((E)-N'-(4-cyanobenzylidene)-N-methylbenzenesulfonyl-hydrazide) was obtained in good yields via the synthetic methodology
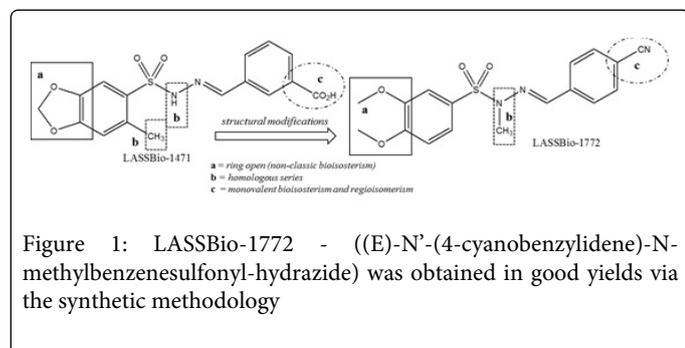
Methods

Synthesis and Characterization of E)-N'-(4-cyanobenzylidene)-N-methylbenzenesulfonylhydrazide (LASSBio-1772)

LASSBio-1772 was obtained in three linear steps using 3,4-dimethoxybenzene-1-sulfonyl chloride as the commercial starting material, employing the methodology previously described by Zapata-Sudo et al. [11] and Kummerle et al. [15], as a pale yellow solid, m.p. 138°C. The purity of LASSBio-1772 was determined by HPLC using a Shimadzu – LC20AD apparatus, a Kromasil 100-5C18 (4,6 mm/6250 mm) column, and the SPD-M20A detector (Diode Array) at 254 nm for quantification of analyte in a 1 mL/min constant flux. The injector was programmed to inject a volume of 20 µL. The mobile phase used was $CH_3CN:H2O$ (6:4) ^{1}H NMR (400 MHz, DMSO-d6) δ (ppm) 7.90 (d, 2H, J=8 Hz, H3'); 7.86 (s, 1H, N=CH); 7.83 (d, 2H, J = 10 Hz, H2'); 7.45 (d, 1H, J=10 Hz, H6); 7.28 (s, 1H, H2); 7.15 (d, 1H, J=8 Hz, H5); 3.81 (s, 3H, 4-OCH3); 3.77 (s, 3H, 3-OCH3); 3.21 (s, 3H, NCH3). ^{13}C NMR (50 MHz, DMSO-d6) δ (ppm) 153.0 (C4); 148.6 (C3); 141.5 (C=N); 138.6 (C1'); 132.8 (C3'); 127.3 (C2'); 127.0 (C1); 121.6 (C≡N); 118.6 (C6); 111.7 (C5); 111.3 (C4'); 110.1 (C2); 55.8 (4-OCH3); 55.8 (3-OCH3); 33.46 (NCH3). HPLC: 98.5 % purity (R.T.=7.00; CH$_3$)

Molecular modeling

The compounds LASSBio-1471 and LASSBio-1772, both E diastereoisomers, were constructed and submitted for conformational analysis by molecular mechanics (MMFF method) with the Spartan 08' (Wavefunction Inc.; License number: DQAIR/HASPUSB). The carboxylic group of LASSBio-1471 was modeled in the ionization state at physiological pH. The most stable conformer of each compound was re-optimized with the AM1 semi-empirical molecular orbital method [19] and saved as mol2 files for docking studies into the Ligand Binding Domain (LBD) of the PPAR-γ crystallographic structure available in the Protein Data Bank (Code 1FM6; resolution: 2.10Å)[20].

The docking studies were implemented with the GOLD 5.1 program (CCDC; License number: G/414/2006) and the rosiglitazone structure was removed from the crystallographic structure (PDB code: 1FM6) to perform docking runs with the ChemPLP fitness function

[21] selected after the re-docking test. Data and poses were analyzed on the Pymol program (license number: 8588).

Induction of experimental diabetes

Male Wistar rats (180–220 g) received a single intravenous injection of streptozotocin (STZ, 60 mg/kg) which was dissolved in citrate buffer (pH 4.5) to induce diabetes. After 4 weeks, the STZ-treated rats were randomly divided into two groups (n=6 each) treated with vehicle (dimethylsulfoxide, DMSO) or LASSBio-1772 (50 mg/kg, i.p.). These groups were compared with a control group of normal rats (non-diabetic).

Measurement of glucose, insulin, triglycerides and total cholesterol in plasma

Plasma glucose levels were examined using the commercial kit, Accu-Check® Performa (Roche, Mannheim, Germany). Four weeks after the induction of diabetes with STZ, rats with glucose levels > 300 mg/dL were intraperitoneally injected with one dose of either vehicle or LASSBio-1772 daily for 7 days. Before and at the end of the treatment period with LASSBio-1772, plasma levels of glucose, insulin, total cholesterol, and triglycerides were measured in diabetic rats. Insulin, total cholesterol, and triglycerides were determined using colorimetric kits (Bioclin, MG, Brazil).

Somatosensory testing – heat withdrawal and allodynia

Heat withdrawal latency was assessed by applying radiant heat to the hind paw using a plantar analgesia meter (model 33, ITC Inc., CA, USA). Control latency was determined using the average response of three measurements separated by 5-min intervals. Mechanical allodynia was determined by the withdrawal threshold to pressure applied to the hind paw (in g) using a digital analgesimeter (model EFF301, Insight, SP, Brazil). Mechanical allodynia and thermal hyperalgesia were evaluated before and weekly after the STZ injection for 4 weeks, as well 3 and 7 days after LASSBio-1772 treatment.

Chemicals

Streptozotocin and carrageenan were purchased from Sigma Chemical (St. Louis, MO, USA). LASSBio-1772 was synthesized and provided by Laboratório de Avaliação e Síntese de Substâncias Bioativas (UFRJ, Brazil). The compound was dissolved in dimethylsulfoxide (DMSO) (Merck, Darmstadt, Germany).
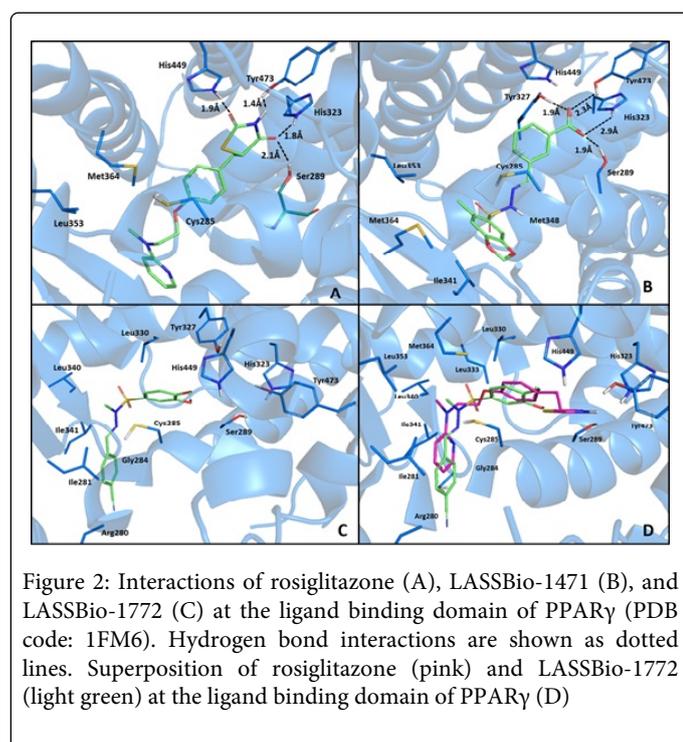
Statistical Analysis

Data were expressed as mean ± S.E.M. and analyzed using the GraphPrism software (version 4.0). Comparison between non-diabetic, diabetic + vehicle (DMSO) and diabetic + LASSBio-1772 groups were analyzed using two-way ANOVA followed by the Bonferroni post-test. Differences between diabetic + vehicle (DMSO) and diabetic + LASSBio-1772 groups were determined using Student t test, with $P<0.05$ considered significant for all tests.

Results and Discussion

Molecular docking studies with PPARγ

As shown in Figure 1, rosiglitazone and LASSBio-1471 were able to interact by hydrogen bonding with Tyr473, His323, and Ser289 in the

ligand binding domain of PPARγ. The carboxylate anion (COO-) group of LASSBio-1471 interacted with the hydroxyl group of Tyr327 and Tyr473 and made a complementary interaction with His323 and Ser289. The 6-methyl-1,3-benzodioxol subunit shows hydrophobic interactions with Met364, Ile341, and Leu353 (Figure 2B). Those interactions are similar to the one produced by rosiglitazone involving the thiazolidine-2,4-dione unit with His323, His449, Ser289, and Tyr473. The hydrophobic interactions were ascribed to the N-methyl group of rosiglitazone, which interacts with Cys285, Leu353, and Met364 (Figure 2A). The interaction profile for rosiglitazone in the PPARγ binding domain is consistent with a previous report [22]. However, the new analog, LASSBio-1772, lost the electrostatic interaction with PPARγ and was unable to perform hydrogen bonding with the receptor. Nevertheless, it could produce a hydrophobic connection with Ile281, Ile341, Gly284, and Cys285 (Figure 2C). These differences in the molecular recognition of LASSBio-1772 by the PPARγ binding domain are represented in Figure 1D. These findings indicate a ligand profile different from a full PPARγ agonist (rosiglitazone-like) that lacks the hydrogen bond interactions with Tyr 473, His449, and His323, and seems more like the partial agonist, BVT13 [23].

Figure 2: Interactions of rosiglitazone (A), LASSBio-1471 (B), and LASSBio-1772 (C) at the ligand binding domain of PPARγ (PDB code: 1FM6). Hydrogen bond interactions are shown as dotted lines. Superposition of rosiglitazone (pink) and LASSBio-1772 (light green) at the ligand binding domain of PPARγ (D)

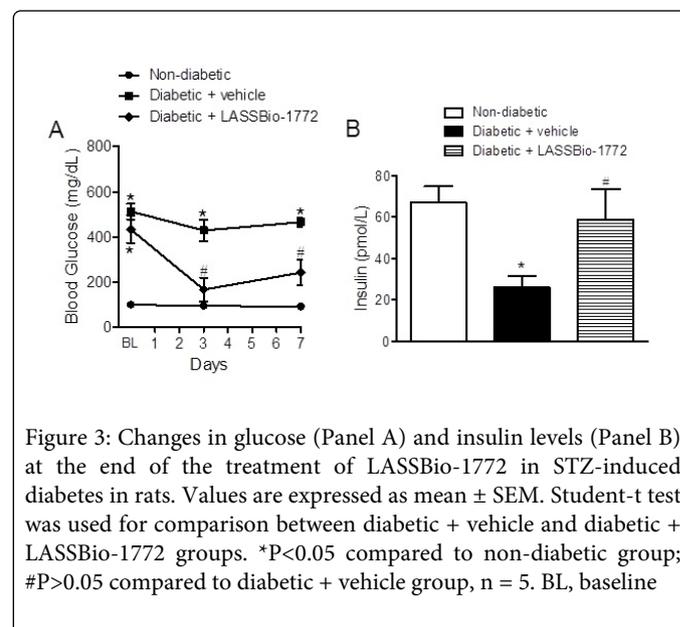## Streptozotocin-induced diabetes in rats

Four weeks after male Wistar rats received an intravenous injection of STZ to induce diabetes, the blood glucose concentration increased from 100.7 ± 5.5 to 512.6 ± 34.7 mg/dL. Serum glucose levels in rats treated with LASSBio-1772 (50 mg/kg, i.p.) were reduced from 433.6 ± 61.2 mg/dL before treatment to 242.4 ± 55.3 mg/dL 7 days after the beginning of treatment (P<0.05) (Figure 3A). Vehicle (DMSO) did not alter glucose levels which was 512.6 ± 34.7 and 410.0 ± 58.0 mg/dL (P=0.16) before and after treatment, respectively. Daily LASSBio-1772 treatment significantly reduced blood glucose levels compared with vehicle-treated diabetic rats, indicating the hypoglycemic activity of this compound. In diabetic rats, insulin levels decreased from $67.1 \pm$

7.7 pmol/L to 25.9 ± 5.6 pmol/L and returned to 58.7 ± 14.8 pmol/L (P<0.05) after 7 days of treatment with LASSBio-1772, indicating that STZ did not cause irreversible damage to the pancreatic islet cells and that LASSBio-1772 increased insulin release.

LASSBio-1772 could directly impact pancreatic -cells by reducing toxicity to the pancreatic islets via an unknown mechanism. Our hypothesis is that LASSBio-1772 may reduce pancreatic inflammation by decreasing the production of inflammatory mediators via activation of PPARγ. In fact, PPARγ agonists such as LASSBio-1772 can impact cytokine production such as tumor necrosis factor a (TNFα) and interleukin-6 [24,25]. Both PPARα and PPARγ receptors regulate inflammatory response [26], but the extent of this regulation and the mechanism are still controversial. LASSBio-1772 may increase insulin levels as well as improve insulin sensitivity via activation of PPARγ to reduce serum glucose levels. Insulin could increase glucose uptake by increasing the concentration of Glut4 proteins. For example, PPARγ activation by rosiglitazone increased the expression and surface translocation of the glucose transporters, GLUT4 and GLUT1, thus increasing glucose uptake in adipocytes and muscle cells and reducing glucose plasma levels [27].

LASSBio-1772 and lipid profile

Diabetic rats showed a significant increase in triglyceride levels, from 49.4 ± 3.5 mg/dL to 79.1 ± 8.5 mg/dL, and LASSBio-1772 restored that dyslipidemia to 33.9 ± 6.3 mg/dL (Figure 4A). There was no change in total cholesterol levels after the induction of diabetes, with levels 57.3 ± 1.3 mg/dl before and 52.5 ± 1.9 mg/dL (P=0.13) after STZ administration. However, LASSBio-1772 treatment reduced cholesterol to 36.7 ± 2.3 mg/dL (Figure 4B).

Figure 3: Changes in glucose (Panel A) and insulin levels (Panel B) at the end of the treatment of LASSBio-1772 in STZ-induced diabetes in rats. Values are expressed as mean ± SEM. Student-t test was used for comparison between diabetic + vehicle and diabetic + LASSBio-1772 groups. *P<0.05 compared to non-diabetic group; #P>0.05 compared to diabetic + vehicle group, n = 5. BL, baseline

LASSBio-1772 may improve lipid profiles in diabetic rats via increasing the lipolysis of circulating triglycerides, as reported for other PPARγ agonists [28].
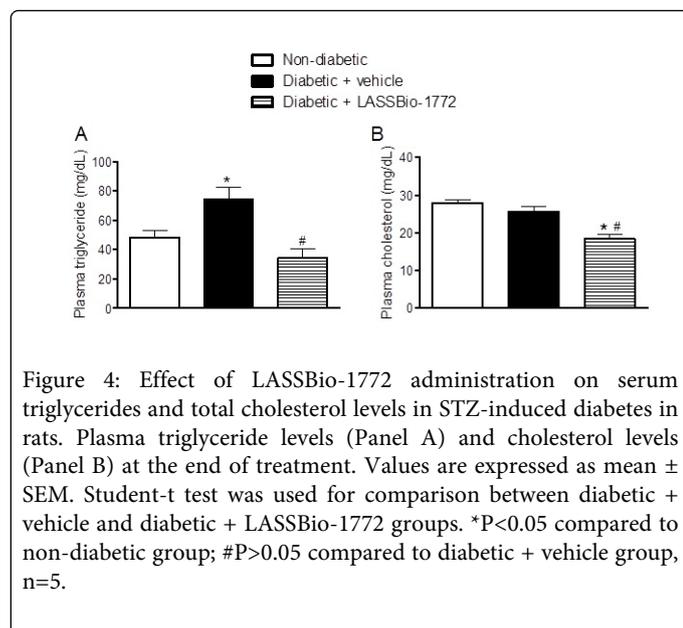
Figure 4: Effect of LASSBio-1772 administration on serum triglycerides and total cholesterol levels in STZ-induced diabetes in rats. Plasma triglyceride levels (Panel A) and cholesterol levels (Panel B) at the end of treatment. Values are expressed as mean ± SEM. Student-t test was used for comparison between diabetic + vehicle and diabetic + LASSBio-1772 groups. *$P<0.05$ compared to non-diabetic group; #$P>0.05$ compared to diabetic + vehicle group, n=5.

Pancreatic sections after treatment with LASSBio-1772

Histological evaluation was done in non-diabetic, diabetic animals treated or not with LASSBio-1772. Normal architecture of islet cells was observed in sections of pancreas of non-diabetic animals (Figure 5A). However, islets of Langerhans of diabetic group were dilated, degranulated and lost their arrangement of dense cords (Figure 5B). In contrast, diabetic rats treated with LASSBio-1772 partially restored the islets cells changes showing less damage and reduction in vacuolation (Figure 5C).
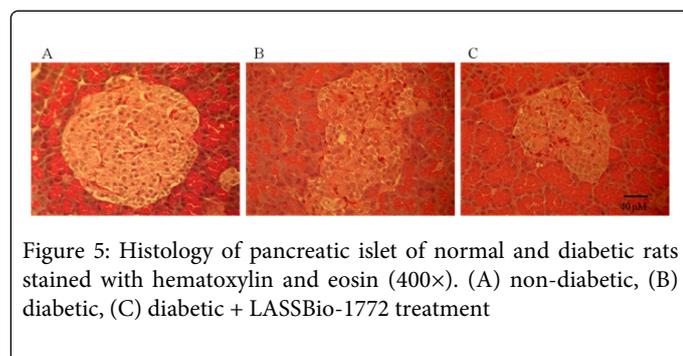

Figure 5: Histology of pancreatic islet of normal and diabetic rats stained with hematoxylin and eosin (400×). (A) non-diabetic, (B) diabetic, (C) diabetic + LASSBio-1772 treatment

Diabetic rats treated with LASSBio-1772 showed less change in pancreas when compared to non-treated group indicating reduction of the degeneration observed after STZ-induced diabetes. The improvement of the architecture of pancreas induced by LASSBio-1772 could stimulate the secretion of insulin from the regenerated islet cells.

LASSBio-1772 reduced pain induced by diabetic neuropathy

Hyperalgesia, defined as decreased withdrawal latency, occurred 4 weeks after diabetes induction (Figure 6A), with change in withdrawal latency from 12.2 ± 0.4 s to 7.1 ± 0.1 s (P<0.01) in the diabetic group. LASSBio-1772 restored these latency values to 12.4 ± 0.5 s after 7 days of treatment. Mechanical allodynia was evaluated using the paw

withdrawal threshold test, and was significantly reduced from 39.2 ± 0.9 g to 29.5 ± 1.0 g in diabetic rats (Figure 6B).
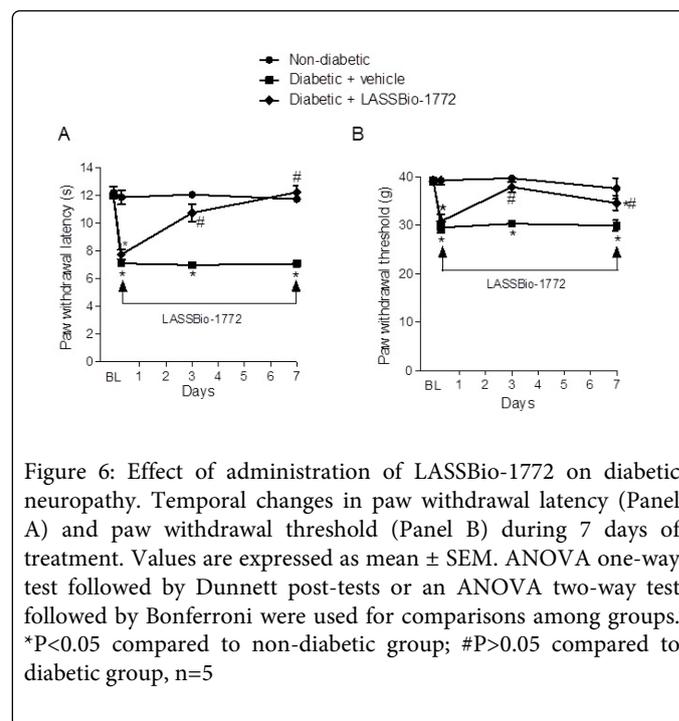

Figure 6: Effect of administration of LASSBio-1772 on diabetic neuropathy. Temporal changes in paw withdrawal latency (Panel A) and paw withdrawal threshold (Panel B) during 7 days of treatment. Values are expressed as mean ± SEM. ANOVA one-way test followed by Dunnett post-tests or an ANOVA two-way test followed by Bonferroni were used for comparisons among groups. *$P<0.05$ compared to non-diabetic group; #$P>0.05$ compared to diabetic group, n=5

Thus, animals in the STZ-induced diabetes group showed evidence of diabetic neuropathy 4 weeks after STZ treatment as evidenced by the appearance of both hyperalgesia and allodynia. LASSBio-1772 significantly increased the paw withdrawal threshold from 30.8 ± 1.1 g (before treatment initiation) to 34.5 ± 1.5 g (Figure 4B), indicating alleviation of the allodynia response. LASSBio-1772 treatment also improved thermal hyperalgesia responses in diabetic animals.

Despite advances in the pharmacologic management of diabetes-induced neuropathic pain, treatment is not effective in all patients. Neuropathic pain observed after peripheral nerve injury, which can be subsequent to prolonged hyperglycemia, is associated with activated microglia in the dorsal horn of the spinal cord [27,28]. Activation of spinal microglia increases the production of pro-inflammatory cytokines, which increase spontaneous nociceptor activity [29]. PPARγ stimulation promotes pain relief, which may be mediated by central sensitization through reduced activation of microglia [28]. Thus, PPARγ agonists such as pioglitazone and rosiglitazone prevent inflammation, which may underlie their neuroprotective mechanism as well. Additionally, these agonists decrease oxidative stress and inhibit nitric oxide generation via reducing the expression of nitric oxide synthase [30]. Therefore, PPARγ is an alternative target for diabetes-induced neuropathy that regulates lipid and glucose metabolism and suppresses inflammation in macrophages [31,32] and microglia. Although the demonstration of the beneficial effects of LASSBio-1772 to reduce hyperglycemia and pain in STZ-induced diabetes, further studies are needed to identify possible side effects which could potentially limit its clinical use.

Conclusions

The new compound, LASSBio-1772, is a PPARγ ligand that restores glucose levels and improve lipid profile in diabetic rats. This partial

agonist of PPARγ is a promising therapeutic candidate for the treatment of neuropathic pain that reduced thermal hyperalgesia and mechanical allodynia in a diabetic neuropathy model.

Acknowledgement

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