

## Design, Biological Evaluation, and PPAR $\gamma$ Modulation of Nitro- and Acetamidophenoxyisobutyric Acids as Antidiabetic Agents

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### ABSTRACT

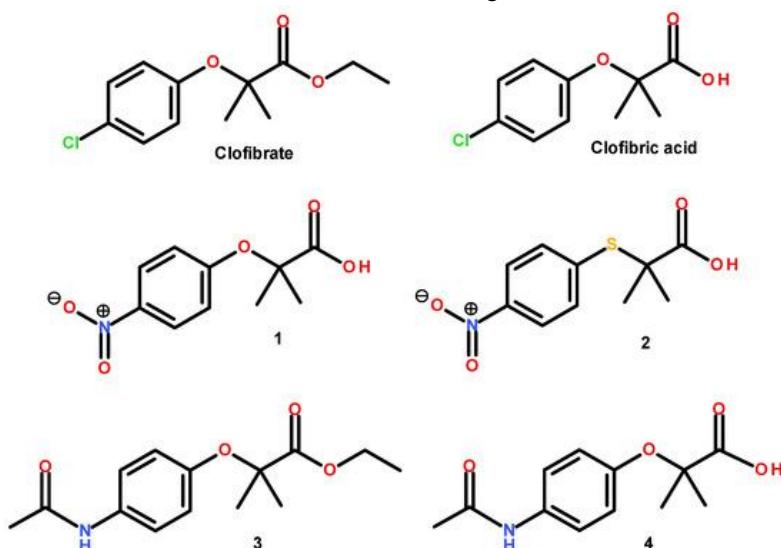
Four isobutyric acid derivatives, including two nitro- and two acetamido-substituted analogues, were synthesized through a two-step procedure and fully characterized by spectroscopic techniques. Treatment of 3T3-L1 adipocytes with compounds 1–4 resulted in elevated mRNA expression levels of PPAR $\gamma$  and GLUT-4, two well-established molecular targets in diabetes therapy, whereas no detectable induction of PPAR $\alpha$  expression was observed *in vitro*. Computational docking and molecular dynamics simulations supported favorable binding interactions between the synthesized molecules and PPAR $\gamma$ . *In vivo* evaluations demonstrated that all tested compounds exerted antihyperglycemic effects linked to enhanced insulin sensitivity. Among the series, nitrocompound 2 exhibited the most pronounced activity and oral bioavailability. Its mechanism of action may involve selective modulation of PPAR $\gamma$ , potentially facilitated by an additional stabilizing interaction with the Gln-286 residue. Overall, these findings identify nitrocompound 2 as a promising experimental candidate for antidiabetic drug development.

**Keywords:** Molecular dynamics, Drug design, PPAR, Diabetes

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### Introduction

Phenoxyisobutyric acid-based compounds constitute the fibrate family of lipid-lowering agents and are primarily recognized for their ability to modulate peroxisome proliferator-activated receptors (PPARs) [1]. The  $\alpha$  and  $\gamma$  isoforms of PPARs interact with endogenous fatty acids and are centrally involved in lipid homeostasis as well as the regulation of insulin sensitivity [2]. Among their downstream effects, PPAR $\gamma$  plays a critical role in controlling genes associated with glucose utilization, including the facilitative glucose transporter GLUT-4 [3]. Clofibrate, the earliest fibrate introduced into clinical practice, is administered as an ester prodrug that is enzymatically hydrolyzed *in vivo* to produce clofibrate acid, the pharmacologically active form responsible for its lipid-lowering properties [4]. Beyond its hypolipidemic action, emerging evidence indicates that clofibrate and its metabolite significantly suppress the activity of 11 $\beta$ -hydroxysteroid dehydrogenase type 1 [5]. This enzyme mediates the regeneration of cortisol from cortisone; elevated cortisol levels antagonize insulin signaling and enhance hepatic glucose production, ultimately contributing to hyperglycemia [6]. Type 2 diabetes mellitus is therefore characterized by persistent elevations in blood glucose resulting from defects in insulin secretion, insulin action, or both [7]. Within the framework of our ongoing efforts to identify novel antidiabetic agents, the present work reports the synthesis of four isobutyric acid derivatives (1–4), (Figure 1), their effects on PPAR $\alpha/\gamma$  activation and GLUT-4 gene expression *in vitro*, computational simulations aimed at predicting their pharmacodynamic behavior, and an evaluation of their antidiabetic efficacy in a diabetic mouse model. The combined application of experimental and computational approaches exemplifies an integrated screening strategy for antidiabetic drug discovery [5].



**Figure 1.** Chemical structures of the PPAR $\alpha$  ligands clofibrate and clofibratic acid, both featuring the phenoxyisobutyric acid core, together with the newly designed nitro derivatives (1–2) and acetamide derivatives (3–4).

## Materials and Methods

### Chemical methods

All starting materials and reagents were sourced from Merck® (Darmstadt, Germany) and utilized without further purification. Proton and carbon nuclear magnetic resonance spectra were acquired using a Varian Oxford spectrometer (Palo Alto, CA, USA) operating at 600 MHz for  $^1\text{H}$  and 150 MHz for  $^{13}\text{C}$ . Molecular weight analysis was carried out on a JMS-700 mass spectrometer (JEOL, Tokyo, Japan) using electron impact ionization. Melting points were determined with an EZ-Melt MPA120 automated melting point apparatus (Stanford Research Systems, Sunnyvale, CA, USA).

### Synthesis of Compounds 1–4

#### 2-(4-nitrophenoxy)isobutyric acid (1)

4-Nitrophenol (1.0 g, 4.44 mmol) was dissolved in acetonitrile together with potassium carbonate (1.22 g, 8.88 mmol). Ethyl 2-bromo-2-methylpropionate (1.29 g, 6.66 mmol) was added slowly, and the reaction mixture was refluxed for 8 h. After cooling, the mixture was poured into ice-cold water, yielding an oily intermediate. This material was subjected to alkaline hydrolysis using LiOH (5 equiv.) in a THF/MeOH/H<sub>2</sub>O mixture (3:2:1, v/v/v) at ambient temperature for 3 h. Following acidification with 10% HCl and solvent removal, the crude product was extracted with dichloromethane. Drying, filtration, and concentration afforded a yellow solid, which was purified by recrystallization from chloroform to give compound 1 (62.8% yield; m.p. 122.8–124.3 °C) [8, 9]; yield 62.8%.  $^1\text{H}$  NMR (200 MHz, CDCl<sub>3</sub>)  $\delta$ : 1.71 (s, 6H, (CH<sub>3</sub>)<sub>2</sub>), 6.91 (d, 2H, H-2', H-6', J = 9.2 Hz), 8.16 (d, 2H, H-3', H-5', J = 9.6), 10.60 (s, 1H, CO<sub>2</sub>H) ppm.  $^{13}\text{C}$  NMR (50 MHz, CDCl<sub>3</sub>)  $\delta$ : 25.5 ((CH<sub>3</sub>)<sub>2</sub>), 79.8 (C-2), 118.2 (C-2', C-6'), 125.7 (C-3', C-5'), 142.3 (C-4'), 160.6 (C-1'), 179.1 (COOH) ppm. MS/EI: m/z (% rel. int.). 225 (M $^+$ , 1%), 180 (M-45, 100%). Anal. calcd. for C<sub>10</sub>H<sub>11</sub>NO<sub>5</sub>: C, 53.33; H, 4.92; N, 6.22. Found: C, 53.41; H, 4.83; N, 6.28.

#### 2-(4-nitrophenylsulfanyl)isobutyric acid (2)

A suspension of 4-nitrothiophenol (1.0 g, 6.40 mmol) and potassium carbonate (1.94 g, 14.1 mmol) in acetonitrile was treated dropwise with ethyl 2-bromo-2-methylpropionate (1.37 g, 7.04 mmol) and heated under reflux for 6 h. Upon completion, the reaction mixture was quenched with cold water. The resulting crude material was hydrolyzed with LiOH (5 equiv.) in THF/MeOH/H<sub>2</sub>O (3:2:1, v/v/v) for 3 h at room temperature. Acidification and extraction with dichloromethane yielded a yellow solid, which was recrystallized from chloroform to afford compound 2 in 58.9% yield with a melting point of 121.9–123.7 °C [10, 11]; yield 58.9%.  $^1\text{H}$  NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$ : 1.56 (s, 6H, (CH<sub>3</sub>)<sub>2</sub>), 7.65 (dd, 2H, H-2', H-6', J = 2.6, J = 9.6 Hz), 8.17 (dd, 2H, H-3', H-5', J = 2.6, J = 9.6 Hz), 10.60 (s, 1H,

CO<sub>2</sub>H ppm. <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>)  $\delta$ : 25.9 ((CH<sub>3</sub>)<sub>2</sub>), 51.5 (C-2), 123.8 (C-2', C-6'), 136.1 (C-3', C-5'), 140.6 (C-1'), 145.4 (C-4'), 179.9 (COOH) ppm. MS/EI: m/z (% rel. int.). 241 (M<sup>+</sup>, 1%), 196 (M-45, 100%). Anal. calcd. for C<sub>10</sub>H<sub>11</sub>NO<sub>4</sub>S: C, 49.78; H, 4.60; N, 5.81; S, 13.29. Found: C, 49.69; H, 4.60; N, 5.87; S, 13.36.

*Ethyl 2-[4-(acetylamino)phenoxy]isobutyrate (3)*

4-Acetylaminophenol (1.0 g, 6.6 mmol) and potassium carbonate (2.0 g, 14 mmol) were dissolved in dimethyl sulfoxide and preheated at 40 °C. Ethyl 2-bromo-2-methylpropionate (1.45 mL, 9.9 mmol) was then introduced dropwise, and the reaction mixture was heated to reflux (80 °C) for 15 h. Reaction progress was monitored by TLC. After completion, the mixture was filtered, and the solid product was recrystallized from acetone to yield compound 3 as white crystals (44.0% yield; m.p. 90.1–92.3 °C) [12]. yield 44.0%. <sup>1</sup>H NMR (200 MHz; DMSO-d<sub>6</sub>; Me<sub>4</sub>Si)  $\delta$ : 1.17 (3H, t, CH<sub>3</sub>), 1.47 (6H, s, (CH<sub>3</sub>)<sub>2</sub>), 2.00 (3H, s, CH<sub>3</sub>CO), 4.15 (2H, q, CH<sub>2</sub>), 6.75 (2H, d, H-2', H-6', J = 8.7 Hz), 7.45 (2H, d, H-3', H-5', J = 8.7 Hz), 9.83 (1H, bs, N-H) ppm. <sup>13</sup>C NMR (50 MHz, DMSO-d<sub>6</sub>)  $\delta$ : 14.6 (CH<sub>3</sub>), 24.5 (CH<sub>3</sub>CO), 25.7 ((CH<sub>3</sub>)<sub>2</sub>), 61.6 (C-2), 79.5 (CH<sub>2</sub>-O), 120.3 (C-2', C-6'), 120.7 (C-3', C-5'), 134.7 (C-4'), 150.9 (C-1'), 168.4 (NHC=O), 173.7 (O-C=O) ppm. EI-MS: m/z (rel. int.) 265 (M<sup>+</sup>, 25%), 192 (25%), 151 (50%), 109 (100%). Anal. calcd. for C<sub>14</sub>H<sub>19</sub>NO<sub>4</sub>: C, 63.38; H, 7.22; N, 5.28. Found: C, 63.38; H, 7.07; N, 5.53.

*2-(4-nitrophenylsulfanyl)isobutyric acid (2)*

Compound 2 was synthesized by reacting 4-nitrothiophenol (1.0 g, 6.40 mmol) with potassium carbonate (1.94 g, 14.1 mmol) in acetonitrile, followed by the gradual addition of ethyl 2-bromoisobutyrate (1.37 g, 7.04 mmol). The reaction mixture was maintained under reflux conditions for 6 h with continuous stirring. After completion, the mixture was quenched by pouring into chilled water, yielding an oily intermediate.

This crude product was immediately subjected to alkaline hydrolysis using lithium hydroxide (5 equivalents) in a ternary solvent system composed of THF, methanol, and water (3:2:1, v/v/v) and stirred at ambient temperature for 3 h. Subsequent acidification with aqueous hydrochloric acid (10% v/v) and partial solvent evaporation afforded a solid residue. Extraction with dichloromethane (3 × 10 mL), followed by drying over anhydrous sodium sulfate, filtration, and concentration, produced a yellow solid. Recrystallization from chloroform yielded compound 2 as a pure material (58.9% yield), with a melting point of 121.9–123.7 °C [10, 11]; yield 58.9%. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$ : 1.56 (s, 6H, (CH<sub>3</sub>)<sub>2</sub>), 7.65 (dd, 2H, H-2', H-6', J = 2.6, J = 9.6 Hz), 8.17 (dd, 2H, H-3', H-5', J = 2.6, J = 9.6 Hz), 10.60 (s, 1H, CO<sub>2</sub>H) ppm. <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>)  $\delta$ : 25.9 ((CH<sub>3</sub>)<sub>2</sub>), 51.5 (C-2), 123.8 (C-2', C-6'), 136.1 (C-3', C-5'), 140.6 (C-1'), 145.4 (C-4'), 179.9 (COOH) ppm. MS/EI: m/z (% rel. int.). 241 (M<sup>+</sup>, 1%), 196 (M-45, 100%). Anal. calcd. for C<sub>10</sub>H<sub>11</sub>NO<sub>4</sub>S: C, 49.78; H, 4.60; N, 5.81; S, 13.29. Found: C, 49.69; H, 4.60; N, 5.87; S, 13.36.

*Ethyl 2-[4-(acetylamino)phenoxy]isobutyrate (3)*

For the preparation of compound 3, 4-acetylaminophenol (1.0 g, 6.6 mmol) and potassium carbonate (2.0 g, 14 mmol) were dissolved in the minimum required volume of dimethyl sulfoxide and warmed to 40 °C. After 20 min of equilibration, ethyl 2-bromo-2-methylpropionate (1.45 mL, 9.9 mmol) was added slowly. The reaction mixture was then heated under reflux at 80 °C and monitored by TLC until completion (15 h).

Upon cooling, the mixture was filtered, and the crude solid was purified by recrystallization from acetone, affording compound 3 as white crystalline material (44.0% yield; m.p. 90.1–92.3 °C) [12]. yield 44.0%. <sup>1</sup>H NMR (200 MHz; DMSO-d<sub>6</sub>; Me<sub>4</sub>Si)  $\delta$ : 1.17 (3H, t, CH<sub>3</sub>), 1.47 (6H, s, (CH<sub>3</sub>)<sub>2</sub>), 2.00 (3H, s, CH<sub>3</sub>CO), 4.15 (2H, q, CH<sub>2</sub>), 6.75 (2H, d, H-2', H-6', J = 8.7 Hz), 7.45 (2H, d, H-3', H-5', J = 8.7 Hz), 9.83 (1H, bs, N-H) ppm. <sup>13</sup>C NMR (50 MHz, DMSO-d<sub>6</sub>)  $\delta$ : 14.6 (CH<sub>3</sub>), 24.5 (CH<sub>3</sub>CO), 25.7 ((CH<sub>3</sub>)<sub>2</sub>), 61.6 (C-2), 79.5 (CH<sub>2</sub>-O), 120.3 (C-2', C-6'), 120.7 (C-3', C-5'), 134.7 (C-4'), 150.9 (C-1'), 168.4 (NHC=O), 173.7 (O-C=O) ppm. EI-MS: m/z (rel. int.) 265 (M<sup>+</sup>, 25%), 192 (25%), 151 (50%), 109 (100%). Anal. calcd. for C<sub>14</sub>H<sub>19</sub>NO<sub>4</sub>: C, 63.38; H, 7.22; N, 5.28. Found: C, 63.38; H, 7.07; N, 5.53.

*2-(4-acetamidophenoxy)isobutyric acid (4)*

Compound 4 was obtained through selective ester hydrolysis of compound 3 (0.5 g, 1.88 mmol). The reaction was carried out in a THF/H<sub>2</sub>O mixture (5:1, v/v) with lithium hydroxide (3 equivalents) at room temperature for 3 h. Following completion, the mixture was acidified with aqueous HCl (10% v/v), and organic solvents were removed under reduced pressure.

The remaining solid was extracted with dichloromethane ( $3 \times 10$  mL), dried over sodium sulfate, filtered, and concentrated. Recrystallization from methanol afforded compound 4 as a white solid in high yield (83.13%), with a melting point of 164.4–166.3 °C [13]; yield 83.13%.  $^1\text{H}$  NMR (200 MHz; DMSO-d<sub>6</sub>; Me<sub>4</sub>Si)  $\delta$  1.46 (6H, s, (CH<sub>3</sub>)<sub>2</sub>), 2.10 (3H, s, CH<sub>3</sub>CO), 6.78 (2H, d, H-2', H-6', J = 8.7 Hz), 7.44 (2H, d, H-3', H-5', J = 8.7 Hz), 9.83 (2H, bs, N–H, COOH) ppm.  $^{13}\text{C}$  NMR (50 MHz, DMSO-d<sub>6</sub>)  $\delta$ : 23.8 (CH<sub>3</sub>CO), 25.1 ((CH<sub>3</sub>)<sub>2</sub>), 78.7 (C-2), 119.5 (C-2', C-6'), 120.2 (C-3', C-5'), 133.9 (C-4'), 161.8 (C-1'), 167.9 (NHC=O), 175.2 (COOH) ppm. EI-MS: m/z (rel. int.) 237 (M<sup>+</sup>, 5%), 192 (M-45, 100%). Anal. calcd. for C<sub>12</sub>H<sub>15</sub>NO<sub>4</sub>: C, 60.75; H, 6.37; N, 5.90. Found: C, 60.8; H, 6.39; N, 5.85.

### *Biological evaluation*

#### *Quantification of GLUT-4 and PPAR expression*

3T3-L1 fibroblasts were cultured to confluence and differentiated into adipocytes using dexamethasone acetate (0.25  $\mu\text{M}$ ), 3-isobutyl-1-methylxanthine (0.5 mM), and bovine insulin (0.8  $\mu\text{M}$ ). After 10 days, mature adipocytes were exposed for 24 h to cumulative concentrations of compounds 1–4.

Total RNA was isolated, and 2  $\mu\text{g}$  of RNA was reverse-transcribed to cDNA. Quantitative PCR was performed using SYBR Green Master Mix with gene-specific primers for GLUT-4 (NM009204.2), PPAR $\gamma$  (NM011146.1), and PPAR $\alpha$  (NM011144). Expression levels were normalized against the housekeeping gene 36B4 (NM007475.2). Relative gene expression changes were calculated using the  $\Delta\Delta\text{Ct}$  method and graphically represented [14, 15].

### *In vivo antidiabetic evaluation*

#### *Experimental animals*

Male ICR mice ( $25 \pm 5$  g) were housed under controlled conditions (25 °C, 12 h light/dark cycle) with free access to food and water. All experimental procedures complied with Mexican regulations NOM-065-ZOO-1999 and NOM-033-ZOO-2014 and were approved by the Institutional Ethics Committee of the Universidad Autónoma Metropolitana (dictum 1857), in accordance with NIH guidelines.

#### *Acute antidiabetic model*

Diabetes was induced following previously reported protocols [5, 14]. After an 8 h fast, mice received nicotinamide (20 mg/kg, i.p.), followed 15 min later by streptozotocin (100 mg/kg, i.p.) dissolved in citrate buffer (0.05 M, pH 4.5). Animals exhibiting fasting glucose levels above 180 mg/dL were selected.

After a further fasting period, mice (n = 6 per group) were orally administered compounds 1–4 (100 mg/kg), vehicle (10% Tween 80), or reference drugs (glibenclamide, 20 mg/kg; pioglitazone, 30 mg/kg). Blood glucose levels were recorded at baseline and at 1, 3, 5, and 7 h post-administration using a glucometer.

#### *Molecular docking studies*

Docking simulations were conducted using AutoDock Vina [16]. The crystal structure of PPAR $\gamma$  complexed with EHA (PDB ID: 2F4B, resolution 2.07 Å) was retrieved from the Protein Data Bank. Water molecules were removed, hydrogen atoms added, and Gasteiger charges assigned using MGLTools 1.5.4 [17]. Ligand structures were generated, minimized, and ionized using MOE with the MMFF94 force field [18].

The docking grid was centered on the co-crystallized ligand coordinates (8.693, -6.961, 39.672) with dimensions of  $40 \times 40 \times 40$  points and 0.375 Å spacing. One hundred genetic algorithm runs were performed, and poses were clustered using a 2.0 Å RMSD cutoff. Visualization and interaction analyses were carried out using MOE and PyMol.

#### *Docking validation*

Method validation was achieved by redocking EHA into the PPAR $\gamma$  binding site. The resulting RMSD value of 0.53 Å confirmed accurate reproduction of the crystallographic binding mode.

#### *Molecular dynamics simulations*

Ligand–protein complexes involving compounds 1–4, clofibrate acid, pioglitazone, and EHA bound to PPAR $\gamma$  (PDB ID: 2F4B) were subjected to molecular dynamics simulations. These simulations aimed to assess the

stability, conformational behavior, and interaction persistence of the complexes, following a previously established protocol [19].

#### Statistical analysis

Comparisons of glycemic variation percentages, as well as in vitro measurements of PPAR $\gamma$  and GLUT-4 expression, were evaluated using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc multiple comparison test. Experimental data are reported as mean values  $\pm$  standard error of the mean (SEM). A probability value of  $p < 0.05$  was considered statistically significant. All statistical analyses were carried out using GraphPad Prism software (version 5.0).

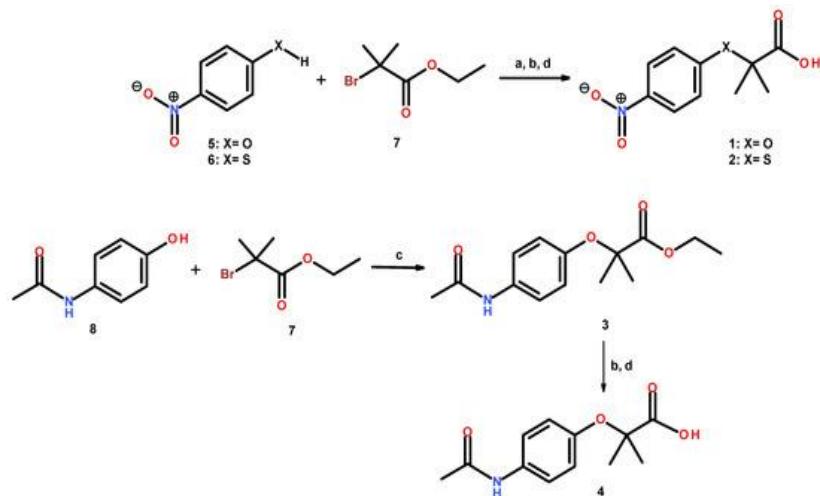
## Results and Discussion

### Chemistry

The synthetic route leading to compounds 1 and 2 is outlined in **Figure 2**. These molecules were obtained through a nucleophilic substitution reaction in which ethyl 2-bromo-2-methylpropionate (7) reacted with either 4-nitrophenol (5) or 4-nitrothiophenol (6). The reactions were carried out under basic conditions using acetonitrile as the reaction medium, yielding the corresponding ethyl ester intermediates as oily residues. Without further purification, these ester intermediates were subjected to hydrolysis using lithium hydroxide (five equivalents) in a mixed solvent system consisting of THF, methanol, and water (3:2:1), resulting in the formation of compounds 1 and 2 with moderate isolated yields of 62.8% and 58.9%, respectively.

Compound 3 was synthesized following an analogous procedure, in which 4-acetylaminophenol served as the nucleophilic reagent. In this case, dimethyl sulfoxide was employed as the solvent under basic conditions, affording compound 3 as a white solid in 44% yield. Subsequent selective cleavage of the ester group using lithium hydroxide produced compound 4 in a significantly higher yield of 83.13% (**Figure 2**).

Reaction progress was routinely monitored by thin-layer chromatography (TLC). Final products were isolated either by filtration or by extraction with dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>), providing compounds 1–4 in overall yields ranging from 44% to 83%. Purification was achieved by recrystallization using appropriate solvents, as detailed in materials and methods section. Structural confirmation of all synthesized compounds was accomplished through spectroscopic techniques, including <sup>1</sup>H and <sup>13</sup>C NMR, as well as mass spectrometric analysis. Elemental microanalysis was performed to verify compound purity.



**Figure 2.** Synthetic pathway for compounds 1–4. Reaction conditions: (a)  $\text{K}_2\text{CO}_3$  in  $\text{CH}_3\text{CN}$  under reflux; (b) hydrolysis with  $\text{LiOH}$  in  $\text{THF}/\text{MeOH}/\text{H}_2\text{O}$ ; (c)  $\text{K}_2\text{CO}_3$  in  $\text{DMSO}$  at  $80^\circ\text{C}$ ; (d) acidic workup with 10% (v/v)  $\text{HCl}$ .

In the <sup>1</sup>H NMR spectra, proton resonances were systematically assigned on the basis of their chemical shifts, signal multiplicities, and coupling constants ( $J$  values). All synthesized compounds exhibited a single resonance in the region of  $\delta_{\text{H}}$  1.46–1.71 ppm, corresponding to the geminal dimethyl moiety. Signals characteristic of para-disubstituted aromatic rings were observed in every molecule. Specifically, the aromatic region displayed a typical

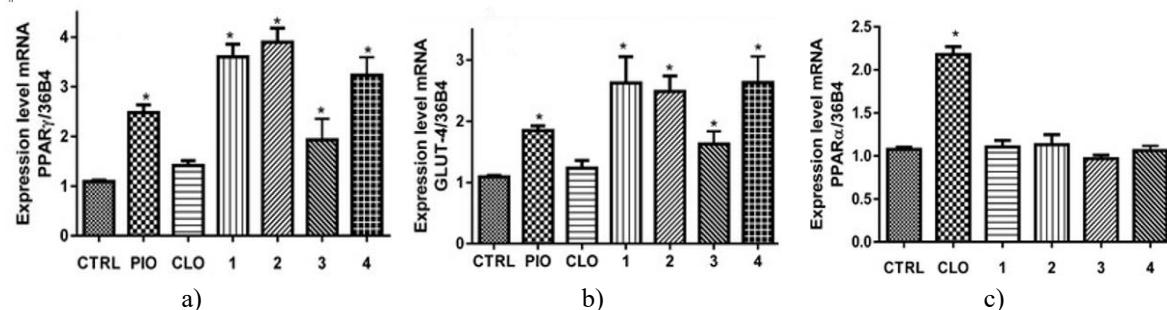
$A_2B_2$  spin system, with doublets appearing between  $\delta_H$  6.75–7.65 ppm ( $J_{\text{ortho}} = 8.7\text{--}9.6$  Hz) and 7.45–8.17 ppm ( $J_{\text{ortho}} = 8.4\text{--}9.6$  Hz), which were assigned to the equivalent H-2'/H-6' and H-3'/H-5' protons, respectively. For compounds 1, 2, and 4, the acidic proton of the carboxyl group appeared as a singlet in the range of  $\delta_H$  9.83–10.6 ppm. In addition, compounds 3 and 4 showed a singlet attributable to the acetamide methyl protons at  $\delta_H$  2.0–2.1 ppm.

Analysis of the  $^{13}\text{C}$  NMR spectra revealed consistent resonance patterns across all compounds. A signal between  $\delta_C$  24.5 and 25.7 ppm was assigned to the geminal dimethyl carbons, while an additional resonance between  $\delta_C$  51.5 and 79.8 ppm corresponded to the C-2 carbon of the ether linkage. In the aromatic carbon region, resonances at  $\delta_C$  118.2–120.3 ppm and 120.2–136.1 ppm were attributed to C-2'/C-6' and C-3'/C-5', respectively. Further downfield signals were observed between  $\delta_C$  133.7–145.4 ppm for C-4', 140.6–161.8 ppm for C-1', and 175.2–179.9 ppm for the carboxylic acid carbonyl carbon. For compounds 3 and 4, additional resonances associated with the acetamide group ( $\text{CH}_3\text{CONH}-$ ) were detected, including methyl carbon signals at  $\delta_C$  23.8–24.5 ppm and carbonyl carbon signals at  $\delta_C$  167.9–168.4 ppm.

#### *In vitro PPAR $\alpha/\gamma$ and GLUT-4 expression*

Prior to gene expression analysis, the cytotoxic potential of compounds 1–4 was assessed in 3T3-L1 fibroblasts using the MTT assay at concentrations of 1, 10, and 100  $\mu\text{M}$ . No reduction in cell viability was detected at any tested concentration. To evaluate the effects of the synthesized compounds on gene expression, murine fibroblasts were induced to differentiate into adipocytes, enabling the assessment of PPAR $\alpha$ , PPAR $\gamma$ , and GLUT-4 mRNA levels. Differentiated cells were exposed to compounds 1–4 at a concentration of 10  $\mu\text{M}$  for 24 h. Pioglitazone (PIO) and clofibrate (CLO) served as reference agonists and were included as positive controls [14, 15].

Quantitative real-time PCR (qPCR) was employed to determine changes in mRNA expression. As illustrated in **Figure 3**, treatment with compounds 1–4 led to a statistically significant increase in PPAR $\gamma$  transcript levels, with an approximate two- to four-fold elevation, accompanied by a three-fold upregulation of its downstream target gene GLUT-4, comparable to the response observed with pioglitazone (PIO). Activation of PPAR $\gamma$  is known to lower blood glucose levels in diabetic patients by improving insulin sensitivity, while increased GLUT-4 expression in skeletal muscle is essential for maintaining glucose balance. Notably, the findings of this study indicate that nitro derivatives 1 and 2, as well as acetamide compound 4, enhanced GLUT-4 mRNA expression to a greater extent than pioglitazone, as shown in **Figure 3b**.



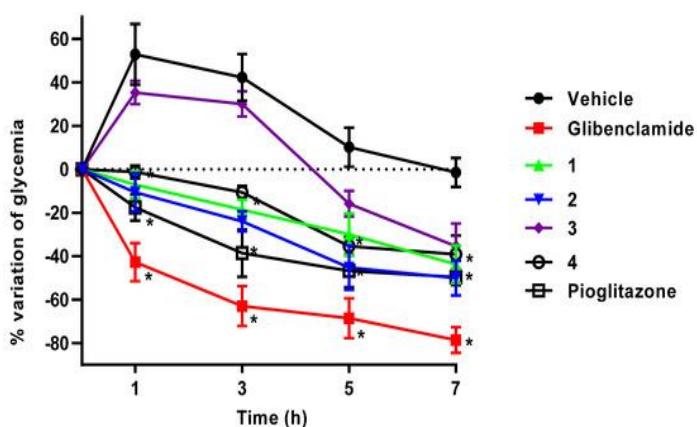
**Figure 3.** Modulation of gene expression levels of PPAR $\gamma$  (a), GLUT-4 (b), and PPAR $\alpha$  (c) following treatment with pioglitazone, clofibrate, and isobutyric acid derivatives 1–4. \* Statistically significant differences between treated groups and the control were determined by one-way ANOVA followed by Dunnett's post hoc test ( $n = 5$ , values expressed as mean  $\pm$  SEM,  $p < 0.001$  versus CTRL).

As anticipated, clofibrate (CLO) failed to induce a significant increase in PPAR $\gamma$  or GLUT-4 mRNA expression. In contrast, none of the synthesized derivatives produced measurable changes in PPAR $\alpha$  transcript levels, whereas clofibrate retained its known activity toward this receptor. These findings indicate that replacement of the chlorine atom on the aromatic ring with aza-containing substituents, such as nitro or acetamide groups, confers preferential activation of PPAR $\gamma$  over PPAR $\alpha$ . Consequently, the synthesized molecules can be classified as selective PPAR modulators (SPPARMs) [20].

#### *In vivo antidiabetic activity*

To validate the hypoglycemic and/or antihyperglycemic potential of compounds 1–4, an acute *in vivo* study was conducted using a mouse model of diabetes induced by streptozotocin (STZ) in combination with nicotinamide (NA). Glibenclamide (Gli) was included as a hypoglycemic reference to confirm partial pancreatic  $\beta$ -cell impairment and to ensure preserved insulin secretion and responsiveness to insulin secretagogues. Pioglitazone was employed as a positive control for antihyperglycemic activity mediated through PPAR $\gamma$  activation. The outcomes of this study are summarized in **Figure 4**.

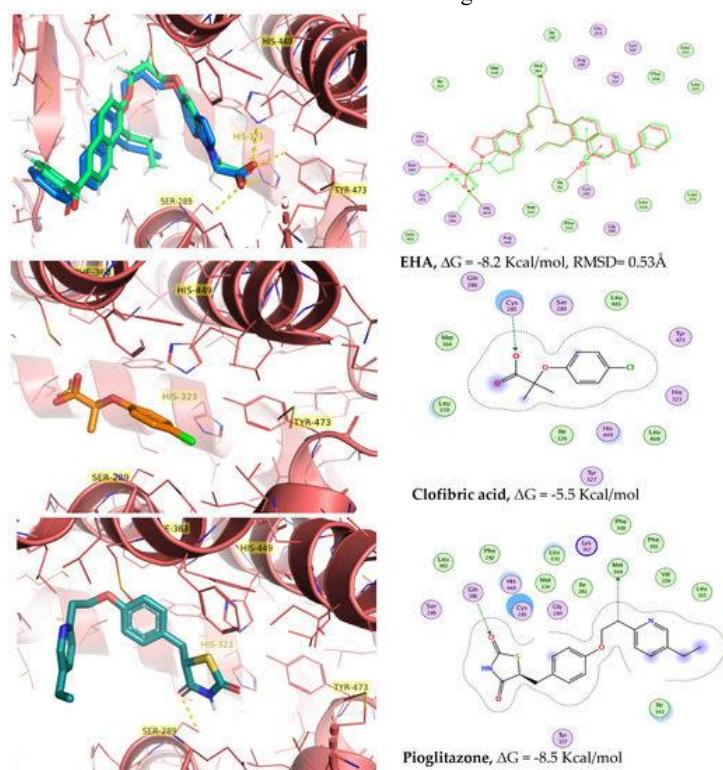
Oral administration of compounds 1, 2, and 4 at a dose of 100 mg/kg via the intragastric route resulted in a pronounced reduction in blood glucose levels within 60 minutes compared to the vehicle-treated group (Tween 80, 10%). This glucose-lowering effect persisted throughout the duration of the experiment. Notably, blood glucose concentrations did not decrease below physiological baseline values, in contrast to the response observed with glibenclamide. This profile indicates an antihyperglycemic mechanism consistent with enhanced insulin sensitivity driven by PPAR $\gamma$  activation [21], as evidenced by the similar glycemic response observed with pioglitazone. Compound 3, however, exhibited a delayed glucose-lowering effect, becoming evident only at 5 and 7 hours after administration. This delayed onset suggests that compound 3 may function as a prodrug of compound 4, requiring metabolic activation through phase I hydrolytic processes *in vivo*.



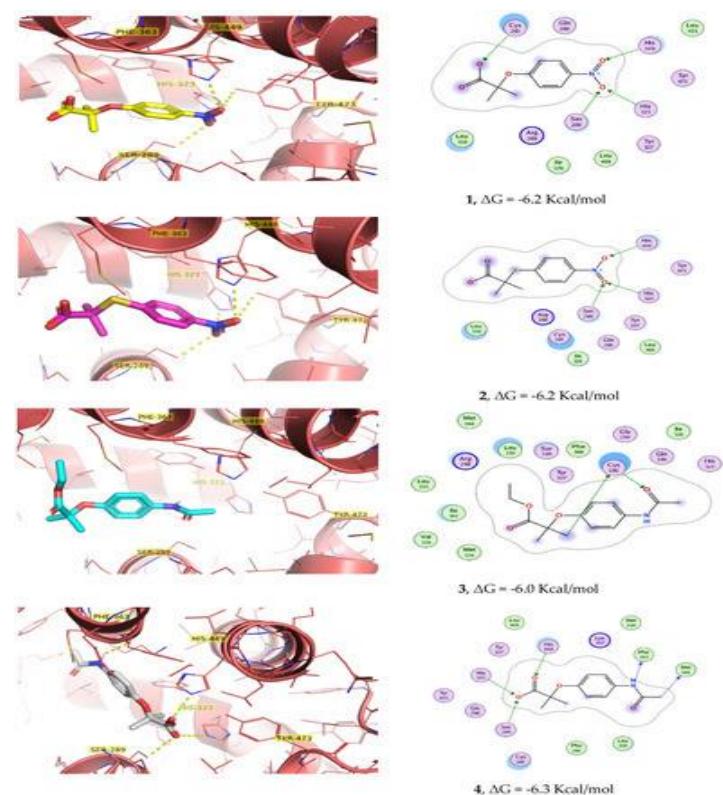
**Figure 4.** Blood glucose response in STZ/NA-induced diabetic mice following a single intragastric dose (100 mg/kg) of (thio)phenoxyisobutyric acid derivatives 1–4, glibenclamide, or pioglitazone. \* Statistical significance versus the control group was determined using one-way ANOVA with Dunnett's multiple comparison test ( $n = 6$ , mean  $\pm$  SEM,  $p < 0.05$ ).

#### Molecular docking analysis

Based on the observed biological effects, including stimulation of PPAR $\gamma$  and GLUT-4 expression *in vitro* and reduction of hyperglycemia *in vivo*, compounds 1–4 were further evaluated using structure-based computational methods. Docking simulations revealed that these derivatives are favorably positioned within the ligand-binding cavity of PPAR $\gamma$  (PDB ID: 2F4B). The predicted binding modes indicated the establishment of a hydrogen-bonding interaction with His-323, accompanied by several stabilizing polar contacts involving Cys-285, Tyr-327, Tyr-473, and His-449, residues that are critically associated with receptor activation (**Figures 5 and 6**). A comparable interaction profile has previously been documented for (5-{3-[(6-benzoyl-1-propyl-2-naphthyl)oxy]propoxy}-1H-indol-1-yl)acetic acid (EHA), a ligand crystallographically resolved in complex with PPAR $\gamma$ , which engages overlapping amino acid residues within the binding site [22]. In contrast, compound 3 failed to establish key receptor interactions, a result that aligns with its comparatively weak biological responses observed in both cellular and animal studies. Of particular note, nitro-bearing compounds 1 and 2 contributed additional polar interactions, specifically involving His-449, His-323, and Ser-289, which may underlie their enhanced activity. Clofibrate acid was included as a reference ligand and docked into the same receptor site. Relative to compounds 1–4, clofibrate acid displayed reduced predicted binding strength toward PPAR $\gamma$ . Validation of the docking protocol yielded a root-mean-square deviation (RMSD) of 0.53 Å<sup>2</sup>, indicating reliable reproduction of ligand positioning. Visualization and analysis of protein–ligand interactions were conducted using PyMol and MOE software tools [18].



**Figure 5.** Three-dimensional binding conformations within the PPAR $\gamma$  ligand-binding domain (PDB ID: 2F4B). The reference ligand EHA is shown as the crystallographic structure (carbon atoms in blue) alongside its redocked conformation (carbon atoms in green). Predicted binding orientations of clofibric acid (carbon atoms in orange) and pioglitazone (carbon atoms in cyan) are also depicted, together with a two-dimensional representation of their key molecular interactions. For EHA, the two-dimensional superimposed interaction maps distinguish the experimental X-ray complex (green) from the redocked model (red).



**Figure 6.** Predicted binding orientations of compounds 1–4 within the PPAR $\gamma$  ligand-binding pocket. Carbon atoms are shown in yellow for compound 1, magenta for compound 2, cyan for compound 3, and gray for

compound 4. Corresponding two-dimensional interaction maps illustrating ligand–receptor contacts are also presented.

Compound 3, which retains its ethyl ester functionality, adopted a binding mode distinct from that of its hydrolyzed analogue 4, present in the ionized carboxylate form. While the aromatic ring of compound 3 was positioned to allow interactions with His-327 and Ser-289, the ester moiety was oriented toward a lateral sub-pocket of the PPAR $\gamma$  binding cavity (Figure 6). The docking-derived binding energies and predicted inhibition constants ( $K_i$ ) for compounds 1–4, together with those obtained for clofibrate, were consistent with the trends observed in both cellular and animal pharmacological evaluations (Table 1). These findings support the conclusion that the glucose-lowering activity of the tested compounds is primarily associated with enhanced insulin sensitivity.

**Table 1.** Docking scores, predicted binding affinities, molecular dynamics–derived binding energies, and quantitative pharmacological parameters for compounds 1–4, pioglitazone and clofibrate acid.

Compound	Predicted Affinity $K_i$ ( $\mu\text{M}$ )	PPAR $\gamma$ Docking Energy $\Delta G$ (kcal/mol)	PPAR $\gamma$ mRNA Induction (Fold Change)	PPAR $\gamma$ MD Binding Energy $\Delta G$ (kcal/mol)	GLUT-4 mRNA Induction (Fold Change)	Maximum Reduction in Blood Glucose (%)
<b>1</b>	5.39	−6.2	3.60	−7.3	2.62	−43.5
<b>2</b>	3.90	−6.2	3.89	−7.8	2.48	−50.1
<b>3</b>	7.54	−6.0	1.92	−7.2	1.62	−35.3
<b>4</b>	4.28	−6.3	3.23	−6.9	2.63	−39.1
<b>Pioglitazone</b>	0.50	−8.5	2.51	−9.7	1.84	−49.6
<b>Clofibrate acid</b>	13.13	−5.5	1.41	−6.8	1.02	No significant effect observed [5]

#### *Molecular dynamics simulations*

Molecular dynamics (MD) simulations were performed to investigate the conformational behavior and stability of the protein–ligand complexes over time. The root-mean-square deviation (RMSD) profiles of both the PPAR $\gamma$  backbone and the bound ligands are presented in Figure 7. Among the analyzed systems, the complex containing nitrocompound 2 reached conformational stability shortly after 2 ns of simulation. Notably, the two most active complexes did not exceed RMSD values of 1.5 Å, indicating highly rigid binding modes. These systems maintained consistent RMSD values throughout the remainder of the simulation, reflecting stable protein–ligand interactions.

As shown, the RMSD trajectories of PPAR $\gamma$  in complex with compound 4 and clofibrate acid exhibited comparable behavior between 8 and 20 ns. In contrast, the backbone RMSD patterns of the EHA- and pioglitazone-bound receptors reflected more pronounced conformational rearrangements, associated with cycles of protein folding and unfolding before reaching a quasi-equilibrium toward the end of the simulation. Analysis of ligand RMSD profiles revealed comparatively lower fluctuations, suggesting limited conformational variability once bound. In particular, compounds 1, 2, and 4 displayed RMSD oscillations centered around 1.5 Å after the first nanosecond, indicating rapid accommodation within the binding pocket and stable anchoring to neighboring amino acid residues previously identified through docking analysis. These observations further support the reliability of the docking predictions.

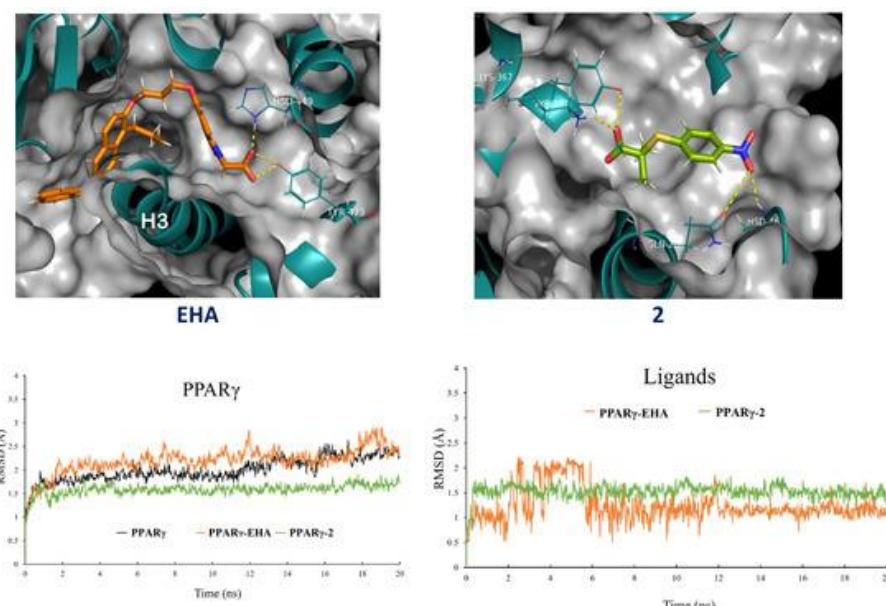
Inspection of Figure 7 indicates that most compounds preserved key anchoring interactions with residues Lys-367, Tyr-327, and His-449. However, nitrocompound 2 exhibited an additional stabilizing interaction involving Gln-286. After approximately 2 ns of simulation, compound 2 established persistent contacts through its nitro group with Gln-286 and His-466, while its carboxylate moiety interacted with Lys-367 and Tyr-327 for the duration of the 20 ns simulation. Unlike the other derivatives, compound 2 induced a noticeable reorientation of the Gln-286 side chain. This residue is known to play a crucial role in stabilizing helices H3 and H12 of PPAR $\gamma$ , thereby exerting a significant influence on receptor activation [23].

Given that partial PPAR $\gamma$  modulators preferentially stabilize the  $\beta$ -sheet region and helix H3 to a greater extent than full agonists [24], nitrocompound 2 may be classified as a partial agonist, potentially offering a reduced side-effect profile. The enhanced stability observed during MD simulations, together with the unique interaction with

Gln-286, is consistent with the strong biological activity of compound 2 observed in both in vitro and in vivo assays.

To further estimate the free binding energies of the PPAR $\gamma$ –ligand complexes, an additional computational approach was applied using the PRODIGY-LIG web server [25]. This platform employs a refinement-based strategy to calculate intermolecular interaction energies and is suitable for diverse protein–ligand systems. The analysis required only the three-dimensional structures of the complexes in PDB format. PRODIGY-LIG predicts binding affinities with a reported root-mean-square error (RMSE) of 1.89 kcal/mol. The resulting binding energy estimates are presented in **Table 1**. Calculations performed for the studied complexes yielded binding free energies comparable to those obtained from molecular docking, albeit slightly more negative, reflecting the stabilizing contribution of molecular dynamics refinement [26].

The design of PPAR-targeted therapeutics that retain efficacy while minimizing the adverse effects associated with full PPAR $\gamma$  agonists remains a central challenge in drug discovery. Consequently, the development of partial PPAR $\gamma$  agonists or selective PPAR $\gamma$  modulators (SPPARMs) represents a promising alternative strategy for the management of metabolic disorders. The compounds described in this study exemplify this approach and demonstrate favorable computational and pharmacological profiles.



**Figure 7.** Comparison of hydrogen-bond interaction patterns formed by EHA (shown in orange) and nitrocompound 2 (shown in green) within the ligand-binding cavity of PPAR $\gamma$  (PDB ID: 2F4B), along with the corresponding RMSD trajectories of the receptor and bound ligands.

#### *In silico toxicological evaluation*

It is widely recognized that nitro-containing compounds are often associated with unpredictable and sometimes severe toxic responses, which has led to their classification as structural alerts and frequent exclusion from drug development pipelines [27]. Nonetheless, nitro functionalities may also serve a dual role, acting not only as pharmacophores but also as selective toxicophores that are linked to tissue-specific toxicity profiles [28]. In order to proactively assess potential safety concerns associated with compounds 1–4, a computational toxicological analysis was undertaken. Predicted toxicity parameters for compounds 1–4, as well as for clofibrate and pioglitazone, were generated using the ACD/ToxSuite software package (version 2.95), and the results are summarized in **Table 2**.

**Table 2.** In silico–estimated toxicity characteristics for compounds 1–4 in comparison with pioglitazone and clofibrate.

Compound	LD <sub>50</sub> Mouse (mg/kg) p.o.	LD <sub>50</sub> Mouse (mg/kg) i.p.	LD <sub>50</sub> Rat (mg/kg) p.o.	LD <sub>50</sub> Rat (mg/kg) i.p.	CYP1A2 Inhibition Probability	CYP3A4 Inhibition Probability	CYP2D6 Inhibition Probability	hERG Blockade Probability
1	1500	680	1590	820	0.00	0.00	0.00	0.01

<b>2</b>	790	670	1280	520	0.00	0.00	0.01	0.01
<b>3</b>	1900	810	3000	770	0.19	0.02	0.01	0.05
<b>4</b>	1400	790	2500	970	0.00	0.00	0.00	0.01
<b>Pioglitazone</b>	1400	400	1100	400	0.02	0.22	0.03	0.10
<b>Clofibrate</b>	1300	750	1800	1200	0.31	0.03	0.04	0.12

Computational predictions of cytochrome P450 inhibition indicated that compounds 1–4 display inhibition profiles for the three major CYP450 isoforms that are comparable to those of clofibrate at clinically relevant concentrations below 10  $\mu$ M. These results suggest a minimal risk of drug–drug interactions and a low likelihood of adverse off-target effects. In addition, all four compounds showed a very low predicted tendency to block the hERG potassium channel at concentrations with  $K_i$  values below 10  $\mu$ M, supporting their classification as non-cardiotoxic agents. Acute toxicity simulations further demonstrated that compounds 1–4 possess  $LD_{50}$  values similar to those predicted for clofibrate and pioglitazone when evaluated via two administration routes across two murine models. Consistent with these in silico findings, experimental assessment of 3T3-L1 fibroblast viability confirmed the absence of cytotoxic effects for compounds 1–4 at concentrations up to 100  $\mu$ M.

The concept of “toxicity cliffs” was first introduced by our group in 2015 [29] and subsequently revisited by other investigators in 2019 [30]. This term describes certain nitro-containing compounds that are flagged by in silico toxicity prediction tools as potentially mutagenic or carcinogenic—such as metronidazole, nimesulide, flutamide, and nitazoxanide—yet exhibit low toxicity in experimental settings. In the present study, compounds 1 and 2 can be classified within this category, as they demonstrate negligible toxic effects despite containing structural motifs commonly identified as toxicity alerts.

To further characterize the drug-likeness of compounds 1–4, their pharmacokinetic behavior was evaluated using the ADMETLab 2.0 platform [31]. Predicted ADME parameters are summarized in **Table 3** and include key absorption-related properties such as gastrointestinal uptake, blood–brain barrier penetration, and oral bioavailability.

**Table 3.** Predicted pharmacokinetic parameters generated using ADMETLab 2.0 (https://admetmesh.scbdd.com/, accessed 5 March 2021) for clofibrate and compounds 1–4.

ADME Category	Parameter	Clofibrate	1	2	3	4
<b>Absorption</b>	Gastrointestinal uptake	High (+)	High (+)	High (+)	High (+)	High (+)
	Predicted oral bioavailability (F)	> 30%	< 20%	> 30%	> 30%	> 30%
	Blood–brain barrier penetration	Yes (+)	No (−)	No (−)	Yes (+)	No (−)
<b>Distribution</b>	Plasma protein binding (%)	97.21	90.62	91.22	60.41	42.14
	Apparent volume of distribution (L/kg)	1.403	0.23	0.32	0.95	0.41
<b>Metabolism</b>	CYP3A4 substrate likelihood	Yes (++)	Yes (+)	Yes (+)	Yes (++)	Yes (+)
	CYP2D6 substrate likelihood	Yes (+)	No (−)	No (−)	Yes (+)	No (−)
<b>Excretion</b>	Total clearance (mL/min/kg)	5.202	0.948	0.412	5.860	1.093
	Predicted half-life (T $_{1/2}$ )	> 3 h	> 3 h	> 3 h	> 3 h	> 3 h

Compounds 1–4 were predicted to possess excellent oral absorption properties, whereas penetration of the blood–brain barrier was limited to compound 3 and the reference drug clofibrate. Drug distribution was evaluated using plasma protein binding and volume of distribution as key indicators. All compounds exhibited protein binding values below the commonly accepted threshold of 95%, suggesting favorable distribution profiles. Nonetheless, compounds with very high protein binding may suffer from a narrow therapeutic margin, as observed for clofibrate.

Predictions related to metabolic behavior indicated that compounds 1–4 are metabolized primarily by cytochrome P450 enzymes, highlighting this enzymatic family as the principal route of biotransformation, consistent with many small-molecule therapeutics. Elimination parameters further demonstrated that these compounds display adequate clearance rates and prolonged elimination half-lives exceeding 3 h, comparable to those calculated for clofibrate.

## Conclusion

This study reports the successful synthesis of four 4-azasubstituted (thio)phenoxyisobutyric acid derivatives and demonstrates their pronounced biological activity. All compounds markedly enhanced the mRNA levels of PPAR $\gamma$  and its downstream target GLUT-4, exceeding the effects observed for the reference drugs clofibrate and pioglitazone. Notably, these derivatives exhibited selective modulation of PPAR $\gamma$  without activation of PPAR $\alpha$ . Structurally, the compounds differ from clofibrate by the replacement of the para-chlorine atom on the aromatic ring with nitrogen-containing substituents, specifically nitro groups in compounds 1 and 2 and acetamide functionalities in compounds 3 and 4. In vivo evaluation revealed that compounds 1, 2, and 4 produced a significant antihyperglycemic response following oral administration in a diabetic animal model. This effect is consistent with an insulin-sensitizing mechanism and supports their classification as selective PPAR $\gamma$  modulators (SPPARMs), comparable to pioglitazone. Among the series, nitro-substituted compound 2 emerged as the most promising candidate. Molecular dynamics simulations highlighted its enhanced stability within the PPAR $\gamma$  ligand-binding domain, attributed to additional interactions involving the nitro moiety and residue Gln-286. These features suggest that compound 2 may function as a partial PPAR $\gamma$  agonist, a profile associated with improved safety and reduced adverse effects.

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**Conflict of Interest:** None

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**Ethics Statement:** None

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