Research Article

Synthesis and In Vitro AMPK Activation of Cycloalkyl/Alkarylbiguanides with Robust In Vivo Antihyperglycemic Action

Erika Gutierrez-Lara,1,2 Carlos Martínez-Conde,1 Edgar Rosales-Ortega,1 Juan José Ramírez-Espinosa,1 Julio C. Rivera-Leyva,1 David Centurión,2 Karla Carvajal,3 Daniel Ortega-Cuellar,3 Samuel Estrada-Soto,1 and Gabriel Navarrete-Vázquez1

1Facultad de Farmacia, Universidad Autónoma del Estado de Morelos, 62209 Cuernavaca, MOR, Mexico
2Departamento de Farmacobiología, Cinvestav-Coapa, 14330 Ciudad de México, Mexico
3Laboratorio de Nutrición Experimental, Instituto Nacional de Pediatría, 04150 Ciudad de México, Mexico

Correspondence should be addressed to Samuel Estrada-Soto; enoch@uaem.mx and Gabriel Navarrete-Vázquez; gnavarrete@uaem.mx

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This work describes the design, synthesis in one step, and the in vitro, in vivo, and in silico antidiabetic evaluation of a series of ten alicyclic and aromatic (alkyl +aryl: alkaryl)biguanides, analogues of metformin and phenformin. The design was conceived using isosteric replacement, chain-ring transformation, and lower and higher homologation strategies. All compounds were obtained as crystals and their structure was confirmed on the basis of their spectral data (NMR and mass spectra), and their purity was ascertained by microanalysis. Compounds were in vitro evaluated as activators of AMP-Activated Protein Kinase (AMPK). The results indicated that compounds 4, 5, and 6 showed similar or even better effect compared to metformin. Docking analysis was performed with regulatory subunit γ of AMPK, showing several interactions with nucleotide binding pocket. The in vivo evaluation of compounds 4–6 at a single dose of 50 mg/kg was performed in a murine experimental model of diabetes. The results showed an important and robust decrease of plasmatic glucose levels (~40%). Compound 6 was selected for an oral glucose tolerance test, showing an antihyperglycemic effect similar to metformin. The in vivo results indicated that compounds 4–6 may be effective in treating experimental T2DM.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a long-lasting and progressive metabolic disease characterized by insulin resistance in several peripheral tissues such as liver, muscle, and adipose, as well as impaired insulin secretion by the pancreas [1]. Metformin (a biguanide, Figure 1) is the most extensively prescribed oral antidiabetic drug for the treatment of T2DM [2]. The main effect of metformin is to decrease hepatic glucose production, being a perfect agent for controlling fasting hyperglycemia. Several mechanisms of action have been proposed, but a previous study [3] reported that metformin increases phosphorylation of the AMP-Activated Protein Kinase (AMPK), with subsequent activation of AMPK activity in hepatocytes. AMPK is a heterotrimeric protein kinase comprised of three subunits: α catalytic subunit and the regulatory β and γ subunits [3]. AMPK is activated by phosphorylation of Thr172 residue on the α-subunit by kinases LKB1 and CaMKKb. Furthermore, AMP binds to nucleotide binding sites on γ-subunits to allosterically activate as well as facilitate Thr172 phosphorylation. AMPK plays a role of “fuel gauge” of the cell as it works to guarantee that ATP levels are maintained under situations of energetic stress such as exercise, hypoxia, and starvation [4]. Due to the central role played by AMPK in cellular energy homeostasis, it has emerged as an attractive drug target for the treatment of a
number of metabolic diseases such as type 2 diabetes mellitus (T2DM) [2].

Another biguanide, phenformin (Figure 1), also activates AMPK, but in 1978 it was retired from the market due to its toxicity, producing lactic acidosis as a major side effect that prompted the withdrawal of phenformin as a treatment for diabetes [5].

In our ongoing research on molecules with antidiabetic activity [6, 7], we report in this manuscript the preparation of ten alicyclic and aromatic biguanides (Table 1), as well as the in vitro activation of AMPK. We also describe the molecular docking of the most active compounds in the nucleotide binding site on γ-subunit of AMPK, and their in vivo anti-hyperglycemic effect using a streptozotocin–nicotinamide rat model of noninsulin dependent diabetes mellitus.

2. Materials and Methods

2.1. Chemicals and Analytical Methods

Starting materials and solvents were purchased from Sigma-Aldrich and were used without any further purification. Melting points were determined using an EZ-Melt MPA120 automated melting point apparatus from Stanford Research Systems and are uncorrected. Reactions were monitored by thin layer chromatography on 0.2 mm precoated silica gel 60 F 254 plates. 1H NMR spectra were recorded on Varian Oxford (400 MHz) and 13C NMR (100 MHz), as well as 1H NMR (200 MHz) and 13C NMR (50 MHz) instruments. Chemical shifts are given in ppm relative to tetramethylsilane (Me4Si, δ = 0) in DMSO-d6 and CDCl3; J values are given in Hz. The following abbreviations are used: s, singlet; d, doublet; q, quartet; dd, doublet of doublet; t, triplet; m, multiplet; bs, broad signal. MS were recorded on a JEOL JMS-700 spectrometer by electronic impact. Element analyses have been carried out on an Elementar Vario ELIII instrument.

2.2. General Procedure for the Synthesis of Compounds 1–10

To a solution of dicyandiamide 21 (0.5 g, 0.0060 mol) in toluene (5 mL) was added 1.1 mol equiv of corresponding alkylamines 11–15 or was added dropwise. After the reaction mixture was stirred at room temperature for 15 min, a mixture 50:50 of HCl diluted in water (2.5 mL) was added dropwise. This mixture was stirred at reflux for 3 to 16 h. After that, the obtained residue was neutralized with a diluted solution of NH4OH. Solvent was removed under vacuum, and the residues were washed with water. The crude solid products were then recrystallized from ethanol affording title compounds (Figure 2, Table 1).

2.2.1. N,N-Diethylimidodicarbonimidic Diame (1). Yield 37%. White crystals obtained from ethanol. Mp 190 °C (Dec). 1H NMR (400 MHz, DMSO-d6) δ: 1.16 (t, 6H, CH3 × 2), 2.89 (q, 4H, N-CH2 × 2), 6.60 (bs, 5H, -NH × 5) ppm. 13C NMR (100 MHz, DMSO-d6) δ: 11.1 (CH3), 41.4 (CH2), 162.9 (C=NH) ppm. MS/El: m/z (% int. rel.) 157 (M+, 25%), 85 (100%). Anal. Calcld for C6H14N2: C, 45.84; H, 9.62; N, 44.55. Found: C, 45.80; H, 9.58; N, 44.57.

2.2.2. N-[Amino(imino)methyl]pyrrolidin-1-carboximidamide (2). Yield 46%. White crystals obtained from ethanol. Mp 156.4–158.7 °C. 1H NMR (400 MHz, DMSO-d6) δ: 1.82 (q, 4H, CH2-CH2), 3.25 (t, 4H, N-CH2 × 2), 6.60 (bs, 5H, -NH × 5) ppm. 13C NMR (100 MHz, DMSO-d6) δ: 23.7 (CH3-CH2), 44.7 (CH2-N), 162.9 (C=NH) ppm. MS/El: m/z (% int. rel.) 155 (M+, 2%), 85 (100%). Anal. Calcld for C6H13N2: C, 46.43; H, 8.44; N, 45.12. Found: C, 46.50; H, 8.48; N, 45.01.

2.2.3. N-[[Amino(imino)methyl][piperidine-1-carboximidamide (3). Yield 63%. White crystals obtained from ethanol. Mp 116.5–118.0 °C. 1H NMR (400 MHz, DMSO-d6) δ: 1.03 (m, 4H, CH2-CH2), 1.05 (m, 2H, CH2), 3.40 (t, 4H, N-CH2 × 2), 6.58 (bs, 5H, -NH × 5) ppm. 13C NMR (100 MHz, DMSO-d6) δ: 18.5 (CH3-CH2), 30.6 (CH2), 35.1 (CH2-N), 162.9 (C=NH) ppm. MS/El: m/z (% int. rel.) 169 (M+, 40%), 85 (100%). Anal. Calcld for C6H13N2: C, 49.68; H, 8.93; N, 41.38. Found: C, 49.62; H, 8.90; N, 41.40.

2.2.4. N-[Amino(imino)methyl]morpholine-4-carboximidamide (4). Yield 22%. White crystals obtained from ethanol. Mp 178.4–180.5 °C. 1H NMR (400 MHz, DMSO-d6) δ: 3.06 (t, 4H, CH2-CH2), 3.75 (t, 4H, N-CH2 × 2), 6.61 (bs, 5H, -NH × 5) ppm. 13C NMR (100 MHz, DMSO-d6) δ: 13.2 (CH3-N), 63.7 (CH2-O), 162.3 (C=NH) ppm. MS/El: m/z (% int. rel.) 171 (M+, 15%), 85 (100%). Anal. Calcld for C6H13N3O: C, 42.09; H, 7.68; N, 40.91. Found: C, 42.10; H, 7.65; N, 40.88.

2.2.5. N-[Amino(imino)methyl]-4-methylpiperazine-1-carboximidamide (5). Yield 54%. White crystals obtained from ethanol. Mp 148 °C (Dec). 1H NMR (400 MHz, DMSO-d6) δ: 2.48 (s, 3H, CH3), 2.73 (m, 4H, CH2-N-CH2), 3.38 (m, 4H, CH2-NMe-CH2), 6.71 (bs, 5H, -NH × 5) ppm. 13C NMR (100 MHz, DMSO-d6) δ: 42.5 (CH2-N), 43.3 (CH3-N), 49.6 (CH3), 163.3 (C=NH) ppm. MS/El: m/z (% int. rel.) 184 (M+, 2%), 85 (100%). Anal. Calcld for C9H16N4O: C, 45.63; H, 8.75; N, 45.61. Found: C, 45.70; H, 8.75; N, 45.58.

2.2.6. N-Benzylimidodicarbonimidic Diamide (6). Yield 56%. White crystals obtained from ethanol. Mp 184.2–186.8 °C. 1H NMR (400 MHz, DMSO-d6) δ: 3.99 (d, 2H, CH2), 7.30 (m, 2H, H-2, H-6), 7.39 (m, 2H, H-3, H-5), 7.50 (m, 1H, H-4), 8.60 (bs, 6H, NH × 6) ppm. 13C NMR (100 MHz, DMSO-d6) δ: 42.6 (CH2-N), 129.4 (C-4), 128.8 (C-2, C-6), 129.0 (C-3, C-5), 134.5 (C-1), 154.9 (C=NH), 156.1 (C=NH) ppm. MS/El: m/z (% int. rel.) 191 (M+, 2%), 91 (100%), 85 (40%). Anal. Calcld for C9H13N2: C, 56.53; H, 6.85; N, 36.62. Found: C, 56.50; H, 6.85; N, 36.61.
2.2.7. N-Phenylimidodicarbonimidic Diamide (7). Yield 75%. White crystals obtained from ethanol. Mp 185.3–187.4°C. 1H NMR (400 MHz, DMSO-d6) δ: 7.19 (m, 5H, H-2 to H-6), 9.91 (bs, 6H, NH × 6) ppm. 13C NMR (100 MHz, DMSO-d6) δ: 121.4 (C-4), 123.7 (C-2, C-6), 129.1 (C-3, C-5), 139.1 (C-1), 155.8 (C=NH), 161.6 (C=NH) ppm. MS/EI: m/z (% int. rel). 177 (M^++, 70%), 119 (100%), 85 (20%). Anal. Calcd for C_8H_11N_5: C, 54.22; H, 6.26; N, 36.90. Found: C, 53.99; H, 6.18; N, 36.91.

2.2.8. N-(4-Nitrophenyl)imidodicarbonimidic Diamide (8). Yield 68%. White crystals obtained from ethanol. Mp 145.5–148.7°C. 1H NMR (400 MHz, DMSO-d6) δ: 7.68 (d, 2H, H-2, H-6, J_o = 9.2 Hz), 8.21 (d, 2H, H-3, H-5, J_o = 9.2 Hz), 11.07 (bs, 6H, NH × 6) ppm. 13C NMR (100 MHz, DMSO-d6) δ: 121.5 (C-2, C-6), 125.5 (C-3, C-5), 142.9 (C-1), 144.5 (C-4), 151.6 (C=NH), 155.4 (C=NH) ppm. MS/EI: m/z (% int. rel). 222 (M^++, 70%), 165 (100%), 85 (10%). Anal. Calcd for C_8H_10N_5O_2: C, 45.40; H, 4.78; N, 37.81. Found: C, 45.38; H, 4.78; N, 37.80.

2.2.9. N-(4-Chlorophenyl)imidodicarbonimidic Diamide (9). Yield 80%. White crystals obtained from ethanol. Mp 161.9–163.3°C. 1H NMR (200 MHz, DMSO-d6) δ: 7.30 (d, 2H, H-2, H-6, J_o = 8.4 Hz), 7.39 (d, 2H, H-3, H-5, J_o = 8.8 Hz), 9.99 (bs, 6H, NH × 6) ppm. 13C NMR (50 MHz, DMSO-d6) δ: 122.5 (C-2, C-6), 127.2 (C-3, C-5), 128.9 (C-4), 136.3 (C-1), 155.3 (C=NH), 161.8 (C=NH) ppm. MS/EI: m/z (% int. rel). 211 (M^++, 90%), 154 (100%), 85 (10%). Anal. Calcd for C_8H_10N_5Cl: C, 45.40; H, 4.78; N, 37.81. Found: C, 45.38; H, 4.78; N, 37.80.

2.2.10. N-(4-Fluorophenyl)imidodicarbonimidic Diamide (10). Yield 85%. White crystals obtained from ethanol. Mp 207.5–209.2°C. 1H NMR (200 MHz, DMSO-d6) δ: 7.18 (m, 2H, H-3, H-5, J_o = 9.2 Hz), 7.42 (m, 2H, H-2, H-6, J_o = 9.2 Hz), 9.91 (bs, 6H, NH × 6) ppm. 13C NMR (50 MHz, DMSO-d6) δ: 117.8 (d, C-3, C-5, J_C-F = 11.25 Hz), 125.6 (d, C-2, C-6, J_C-F = 3.9 Hz), 137.6 (d, C-1, J_C-F = 1.55 Hz), 160.9 (d, C-4, J_C-F = 294.7 Hz), 159.8 (C=NH), 162.1 (C=NH) ppm. MS/EI: m/z (% int. rel). 195 (M^++, 80%), 111 (100%), 85 (10%). Anal. Calcd for C_8H_10N_5F: C, 49.22; H, 5.16; N, 35.88. Found: C, 49.21; H, 5.20; N, 35.90.

2.3. Biological Activity

2.3.1. In Vitro AMPK Activation. Primary rat hepatocytes were obtained by collagenase digestion as described by Berry...
and Friend [8]. Fresh isolated hepatocytes were equally distributed in collagen-coated dishes and incubated at 37 °C for 2 h in medium α MEM (Gibco; cat i900-024), 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. The cultured dishes were then washed three times with PBS to remove unattached dead cells. Dishes were randomly assigned to receive fresh FBS-free medium without or with different concentrations of both metformin (10 mM) and ten of its analogues (1 and 10 mM) for 1 h. After this time, the plated cells were washed three times with cold PBS. The cells were then lysed using a buffer containing 50 mM HEPES (pH 7.5), 50 mM KCl, 1 mM EDTA, 5 mM EGTA, 1 mM glycerolphosphate, 0.1% (vol/vol) Triton X-100, 50 mM NaPPI, 1 mM orthovanadate, and 1 mM DTT, a standard complete protease inhibitor mixture (Roche). The lysates were then centrifuged (10,000 g, 10 min, 4 °C), and the supernatants were stored at −80 °C. The activation of AMPK and its target protein, acetyl-CoA carboxylase (ACC), was determined by immunoblot detection with antibodies against phospho-Thr172 AMPK and phospho-Ser79 ACC, as well as total AMPK, ACC, and actin as loading controls. All antibodies were purchased from Cell Signaling.

2.3.2. In Vivo Antidiabetic Activity. Male Wistar rats weighing an average of 300 g were used. They were maintained at 25 °C in a 12 h light/dark cycle and at 45–65% of humidity during experimentation time. All animal procedures were developed in accordance with the Mexican Federal Regulations for Animal Experimentation and Care, ratified by the Institutional Animal Care and Use Committee (UNAM) based on US National Institute of Health Publication #85-23 [11, 12].

2.3.3. Induction of Diabetes. Streptozotocin (STZ) was dissolved in citrate buffer (pH 4.5) and nicotinamide was dissolved in normal physiological saline solution. Non-insulin-dependent diabetes rat model was induced in overnight fasted rats by a single intraperitoneal injection of 100 mg/kg STZ, 15 min before the i.p. administration of 40 mg/kg of nicotinamide. Hyperglycemia was confirmed by elevated glucose concentration in plasma, determined after 2 weeks by strip-glucometer. The animals with blood glucose concentration higher than 200 mg/dL were used for the antidiabetic study [11, 12].

2.3.4. Non-Insulin-Dependent Diabetes Mellitus Rat Model. The diabetic animals were divided into three groups of five animals each (n = 5). Rats of experimental groups were assigned to receive fresh FBS-free medium without or with 1, 1.5, 2, 2.5, and 3 h after vehicle, of solution. Blood samples were collected from the tail tip at 0 (before oral administration), 1, 1.5, 2, 2.5, and 3 h after vehicle, positive control, or test compound administration.

2.4. Docking Studies. Discovery Studio version 3.5 and Pymol version 1.0 were used for visualization. The crystal structure of AMPK was retrieved from the PDB with the accession code: 2UV4. Docking calculations were conducted with AutoDock Vina. The program performs several runs in each docking experiment. Each run provides one predicted binding mode. All water molecules and also cocrystal ligand (5’-adenylic acid) were removed from the crystallographic structure. The AutoDock Vina plugin through Pymol program was used, where we generated the grid maps. Each grid was centered at the crystallographic coordinates of the cocrystal ligand. The grid dimensions were 20 × 20 × 20 Å³ with points separated by 1.0 Å. Also, the protein file was selected as the rigid part, and the ligand file as the flexible one, allowing all its torsions to rotate during docking. AutoDock Vina uses default algorithms of searching and automatically prepares the files for use as it adds charges and polar hydrogens to the protein necessary to perform scoring calculations; it clusters showing only the main results. The number of docking runs was 10. After finishing, the poses were visualized on Pymol and compared against the cocrystallized ligand over the protein.

2.4.1. Docking Validation. The molecular docking protocol was validated by redocking of cocrystal ligand (5’-adenylic acid) into the active site of the structure of AMPK. The root mean square deviation between the cocrystal ligand and the docked structure was less than 2.5 Å. This value indicates that the parameters for docking simulations are good in reproducing orientation and conformation in the X-ray crystal structure of enzyme and receptors.

3. Results and Discussion

3.1. Chemistry. Compounds 1–10 were designed on the basis of the structure of metformin and phenformin (Figure 1,
Table 1), maintaining the biguanide group, removing both dimethyl and phenylethyl side chains, and substituting the proximal amino group with diethyl or cycloalkyl groups using a straightforward approach called chain-ring transformation, attaining conformational constraint connecting alkyl substituents to give the corresponding cyclic analogues. The homologation criteria were employed to pass from pyrrolidine to piperidine. A homologous series is a group of compounds that differ by a constant unit, generally a methylene group [14]. Morpholine and 4-methylpiperazine derivatives were selected as isosteric replacements of piperidine. Benzyl or 4-substituted phenyl groups were designed as lower homologues, with one or two methylene groups less than those presented by phenformin. Some physicochemical properties of compounds 1–10 are described in Table 1.

Compounds 1–10 were prepared in a single step starting from cyanoguanidine, which was condensed with several alkylamines 11–15 or aryl amines 16–20 under reflux conditions (Figure 2). Title compounds were recovered with 22–85% yields and purified by recrystallization with ethanol. Their chemical structures were confirmed by spectral data (NMR and mass spectra), and their purity was ascertained by elemental analysis.

3.2. In Vitro Biological Activity. To test the ability of each derivative to activate AMPK, an in vitro assay was performed on a primary culture of hepatocytes. Aliquots of stock solutions of the analogues (dissolved in DMSO) were diluted with the assay buffer, using metformin as positive control. The phosphorylation of AMPK and its target ACC were assessed by immunoblot analysis, where it is observed that aliphatic or alicyclic compounds 1–3 were not able to activate the enzyme (data not shown). Conversely, we found that AMPK phosphorylation (activation) was increased in a concentration-dependent manner with compounds 4, 5, and 6, being more pronounced with compound 6; in fact, the concentrations of three of the compounds required for activation of AMPK were significantly lower than those of metformin (Figure 3). On the other hand, aromatic biguanides 7–10 were unable to activate the AMPK. These results are in accordance with those reported in a parallel work performed with closely related biguanides [15].

In the immunoblot not only is the phosphorylated AMPK observed, but also the phosphorylated ACC (acetyl-CoA carboxylase) in serine 79 can be detected. This enzyme is one of the targets of AMPK. The phosphorylation of this enzyme causes its inactivation, and this leads to an increase in the oxidation of free fatty acids [16]. The phosphorylation of ACC indicates that analogues induce the phosphorylation (activation) of AMPK, which leads to modification of the different metabolic pathways.

3.3. In Silico Studies

3.3.1. Docking Analysis. Based on the in vitro biological assay of AMPK activation, the most active compounds were selected to explain the experimental activities. On this basis, a preliminary molecular docking study was conducted to evaluate the putative binding mode of compounds 4–6 into the regulatory γ-subunit of AMPK. This molecular docking reveals that compounds 4 and 5 internalize into the nucleotide binding pocket of AMPK and interact by electrostatic and hydrogen bonds with Asp-317 and Thr-200, and both residues are essential for the activation of this enzyme (ΔG = 5.2 kcal/mol for both compounds). However, compound 6 (the most active in vitro) showed an
3.3.2. In Silico Toxicity. With the aim of anticipating potential toxicity issues of compounds 4–6, a computational prediction of safety profiles was performed. The toxicity parameters of 4–6, metformin, and phenformin were calculated through the ACD/ToxSuite software, v. 2.95 (Table 2).

The in silico calculation of inhibition for the three main isoforms of CYP450 for compounds 4–6 was comparable to that of metformin at relevant clinical concentrations (<10 μM), showing low probabilities of drug-drug interactions and undesirable adverse effects [17]. Several basic nitrogen compounds are associated with cardiovascular risks due to human ether-a-go-go related gene (hERG) channel blockade [18–20]. Compounds 4–6 showed low prediction of hERG channel blockage at clinically relevant concentrations (Ki < 10 μM), being considered as potentially noncardiotoxic compounds. In the calculation of acute toxicity, compounds 4–6 demonstrated similar predicted LD50 than metformin and phenformin by two different administration routes.

3.4. In Vivo Antidiabetic Effect of Compounds 4–6. Compounds 4–6 were the most potent AMPK activators of the series, and they were selected in order to evaluate their in vivo antidiabetic activity using an STZ-nicotinamide non-insulin-dependent diabetes mellitus rat model. Metformin (50 mg/kg) was used as a positive control. The antidiabetic activity of compounds 4–6 was determined using a 50 mg/kg single dose, by intragastric route (Figure 5).

The antidiabetic assay shows that analogue 4 significantly reduced glucose levels compared to the vehicle and is as good as to the control group (metformin), having at 7 hours after administration a percentage of glucose decrease of 33.5%. analogue 5 also decreased glucose levels compared to
vehicle. In addition, its effect was similar to that presented by metformin at the same dose. The activity was retained during the 7 hours of experimentation. At 7 hours after administration, a 40% decrease in blood glucose levels was observed. Analogue 6, which was the compound that showed the best activity on AMPK activation, was also active in the in vivo assay, and its antihyperglycemic effect was retained throughout the assay.

In order to verify the plausible antihyperglycemic effect of compound 6, glucose tolerance test curves in normoglycemic rats were obtained. Dose of 50 mg/kg for 6 and metformin was employed. As shown in Figure 6, compound 6 displayed a significant reduction of hyperglycemic peak which was attained at 0.5 h after glucose administration. In Figure 6, it can be seen that the animals treated with analogue 6 reached a lower hyperglycemic value than the animals treated with metformin and compared to the vehicle, 0.5 h after the treatment.

During the experiment, glucose levels did not decrease beyond baseline, indicating that the antidiabetic effect of compound 6 is due to an antihyperglycemic action rather than a hypoglycemic effect. Also, compounds 4–6 did not increase the lactic acid concentrations in plasma of rats tested (less than 1.9 mM), so any evidence of lactic acidosis was found. With these results, it can be concluded that the mechanism of action of the analogues 4–6 that confer their antidiabetic activity is similar to metformin, through the activation of AMPK and of some of the pathways that are regulated by this enzyme. Previously in vitro reports agree with the AMPK results obtained with compound 6 [15]. However, in our current study, we have demonstrated the robust in vivo effect produced by this compound after an oral administration. Further studies are being conducted by us in order to demonstrate the cardiovascular action of compound 6 in a murine model of fructose-induced insulin resistance [21].

4. Conclusion

In summary, ten alkarylbiguanides have been developed as promising compounds for the treatment of type 2 diabetes mellitus. Compounds 4–6 (a) exhibited AMPK activation similar to or greater than metformin, (b) demonstrated a robust reduction of glucose levels with marked in vivo antihyperglycemic efficacy, and (c) showed predicted low toxicity profiles and any experimental evidence of lactic acidosis. These compounds could be an alternative to metformin, the only biguanide currently available.

Disclosure

The paper is taken in part from the M. Pharm. thesis of E. Gutierrez-Lara.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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