Supporting Information

Design and Synthesis of Acetylenyl Benzamide Derivatives as Novel Glucokinase Activators for the Treatment of T2DM

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1. Chemistry

All reagents and solvents were used as received from commercial sources. ^{1}H NMR spectra were recorded on a Bruker, Avance 400 Spectrometer 400 MHz nuclear magnetic resonance spectrometer. ^{1}H NMR spectra were recorded in $CDCl_3$, CD_3OD, or DMSO-d_6, and chemical shifts are reported relative to a TMS internal standard to the residual solvent peak (abbreviation in spectra: s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet, m = multiplet). UPLC analysis was performed using an Acquity UPLC BEH○R C18 (1.7 μm, 2.1 × 100 mm) with 5% acetonitrile in water (0–1 min), 15–90% acetonitrile in water (1–8.5 min), 90% acetonitrile (8.5–10 min), with both solvents containing 0.1% formic acid as a modifier with a flow of 0.4 mL/min; and UV detection at 230 nm. Purification by flash chromatography was carried out using either Zeochem C-gel 560 (0.060–0.200 mm, Zeochem) or MPLC with a Teledyne Isco CombiFlash Rf with RediSep Flash columns using a gradient of ethyl acetate in n-hexanes or methanol in CH_2Cl_2, or similar instrument or reverse phase preparative HPLC. TLC analysis was performed on silica gel 60 F_{254} plates.

Liquid chromatography mass spectra (LCMS) were obtained on Agilent technologies G6120B using electrospray ionization (ESI). LCMS analysis was performed using a Eclipse Plus C18 (3.5 μm, 4.6 × 100 mm) with 46–91% acetonitrile in water (0–2.5 min), 91% acetonitrile (2.5–4.0 min), 91-46% acetonitrile in water (4.0-4.5 min) and staying at 46% acetonitrle (4.5-5.0 min), with both solvents containing 0.1% formic acid as modifier with a flow of 1.5 mL/min and UV detection at 230 nm. Purity of all final analogues for biological testing were confirmed to be >95% as determined by UPLC analysis and inspection of NMR spectra.

1.1. Synthesis of 3-Bromo-5-(2-methoxy-(1*S*)-methyl-ethoxy)-benzoic acid methyl ester (8)

To a solution of 3-bromo-5-hydroxybenzoic acid (25.0 g, 0.12 mol) in methanol (250 mL) were added conc. H_2SO_4 (1.0 mL). The reaction mixture was refluxed overnight. The reaction mixture was cooled to room temperature, and then concentrated under reduced pressure. The reaction mixture was added ethyl acetate and washed with water. The organic layer was dried over $MgSO_4$, concentrated under reduced pressure, and purified by flash column chromatography (EA:Pet.Ether = 1:5) to afford 18.5 g as a white solid.

^{1}H NMR (400MHz, DMSO) δ 10.36 (s, 1H), 7.48-7.47 (t, 1H), 7.33-7.32 (q, 1H), 7.22-7.21 (t, 1H), 3.83 (s, 3H); TLC R_f 0.70 (20% EtOAc in Pet.Ether).

To a mixture of 3-bromo-5-hydroxy-benzoic acid methyl ester (18.5 g, 0.08 mol), (R)-(-)-1-methoxy-2-propanol (10.2 g, 0.11 mol) and triphenylphosphine (29.8 g, 0.11 mol) in tetrahydrofuran (180 mL) was added dropwisely diisopropyl azodicarboxylate (23.0 g, 0.11 mol) at ice bath under nitrogen. The reaction mixture was stirred at room temperature overnight. The reaction mixture was concentrated under reduced pressure and purified by flash column chromatography (EA:Pet.Ether = 1:20) to afford 22.6 g (68% yield) of **8** as a

colorless oil.

^{1}H NMR (400MHz, $CDCl_3$) δ 7.74-7.73 (t, 1H), 7.53-7.52 (q, 1H), 7.28-7.27 (t, 1H), 4.60-4.56 (m, 1H), 3.90 (s, 3H), 3.59-3.54 (q, 1H), 3.50-3.47 (q, 1H), 3.40 (s, 3H), 1.32-1.30(d, 3H); TLC R_f 0.60 (15% EtOAc in Pet.Ether).

1.2. Synthesis of 3-Bromo-5-(2-methoxy-(1*S*)-methyl-ethoxy)-benzoic acid (9)

To a solution of **8** (22.6 g, 0.075 mol) in tetrahydrofuran (200 mL) / methanol (200 mL) were added 3N sodium hydroxide. The reaction mixture was stirred at room temperature overnight. The reaction mixture was concentrated under reduced pressure, and then acidified by 1N hydrochloric acid. The reaction mixture extracted with ethyl acetate. The combined organic layers were washed with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford 20.2 g (92% yield) of **9** as a colorless oil.

^{1}H NMR (400MHz, $CDCl_3$) δ 10.6 (brs, 1H), 7.79-7.78 (t, 1H), 7.57-7.56 (q, 1H), 7.32-7.31 (t, 1H), 4.64-4.57 (m, 1H), 3.62-3.58 (q, 1H), 3.54-3.51(q, 1H), 3.42(s, 3H), 1.33-1.31(d, 3H); TLC R_f 0.50 (30% EtOAc in Pet.Ether).

1.3. Synthesis of 3-Bromo-5-(2-methoxy-(1*S*)-methyl-ethoxy)-*N*-(1-methyl-1*H*-pyrazol-3-yl)-benzamide (10)

To a solution of **9** (20.2 g, 0.069 mol) in dichloromethane (200 mL) were added 1-methyl 1*H*-pyrazol-3-ylamine (11.2 g, 0.115 mol), HOBT (15.5 g, 0.115 mol), EDAC (22.0 g, 0.115 mol) and diisopropylethylamine (29.7 g, 0.229 mol) at room temperature. The reaction mixture was stirred at room temperature overnight. The reaction mixture was added water, dried over $MgSO_4$, filtered and concentrated under reduced pressure, and purified by flash column chromatography (EA:Pet.Ether = 1:10 ~ 1:2) to afford 22.5 g (80% yield) of **12** as a colorless oil.

^{1}H NMR (400MHz, $CDCl_3$) δ 9.72 (s, 1H), 7.51 (s, 1H), 7.37 (s, 1H), 7.30-7.28 (t, 1H), 7.23-

7.22 (t, 1H), 6.85-6.85 (d, 1H), 4.54-4.50 (m, 1H), 3.63 (s, 3H), 3.57-3.52 (q, 1H), 3.49-3.45 (q, 1H), 3.39(s, 3H), 1.29-1.27(d, 3H); TLC R_f 0.40 (50% EtOAc in Pet.Ether); MS m/z for $C_{15}H_{18}BrN_3O_3$ [Br^{79}, M + H]$^+$ calcd 368.1, found 368.0.

1.4. Synthesis of 3-(3-amino-phenylethynyl)-5-(2-methoxy-(1*S*)-methyl-ethoxy)-*N*-(1-methyl-1*H*-pyrazol-3-yl)-benzamide (19)

To a solution of **10** (700 mg, 1.90 mmol) in tetrabutylammonium fluoride trihydrate (3.60 g) was added 3-ethylnylaniline (334 mg, 2.85 mmol) and $PdCl_2(PPh_3)_2$ (70 mg, 0.10 mmol). The reaction mixture was heated at 85 ^oC overnight. The reaction mixture was cooled to room temperature, and then added ethyl acetate. The reaction mixture was washed with water, dried over $MgSO_4$, filtered and concentrated under reduced pressure, and purified by flash column chromatography (EA:Pet.Ether = 1:2) to afford 545 mg (71% yield) of **19** as a yellow oil.

^1H NMR (400MHz, $CDCl_3$) δ 8.71 (brs, 1H), 7.53 (s, 1H), 7.44 (s, 1H), 7.29-7.28 (d, 1H), 7.22-7.22 (d, 1H), 7.16-7.12 (t, 1H), 6.94-6.92 (d, 1H), 6.84-6.82 (t, 2H), 6.69-6.67 (q, 1H), 4.64-4.59 (m, 1H), 3.78(s, 3H), 3.73(brs, 2H), 3.61-3.57(q, 1H), 3.53-3.49(q, 1H), 3.42(s, 3H), 1.33-1.32(d, 3H); MS m/z for $C_{23}H_{24}N_4O_3$ [M + H]$^+$ calcd 405.1, found 405.0.

2. Bioassays

For experiments involving the use of animals, all procedures were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee of Yuhan Research Institute.

2.1. Glucokinase enzymatic assay

An enzymatic glucokinase assay using purified recombinant human pancreatic glucokinase was used to evaluate the compounds. Glucokinase activity was assessed spectrometrically by a coupled reaction with glucose-6-phosphate dehydrogenase

(G6PDH).[34] Briefly, GK catalyzes glucose phosphorylation to generate glucose-6-P, which is oxidized by G6PDH with the concomitant reduction of NADPH. The resultant NADPH is then monitored via an increase in the rate of absorbance at 340 nm using a plate reader (Spectra-Max 384 plus, Molecular Devices, CA, USA). All compounds were prepared in DMSO and the assay was performed in 96-well plates in a final volume of 100 μL containing 25 mM HEPES pH 7.4, 10 mM glucose, 25 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 1 mM NADP, 2.5 U/mL G6PDH, 1 mM glucose, 0.1 μg glucokinase, and the test compounds. The fold activation of the enzyme was assessed by comparing with controls (GK activation in DMSO only was considered as 100%). For EC_{50} determination, 12 different concentrations of the compounds were tested in the assay, and the fold changes in activity versus controls were fitted to a sigmoidal curve using a dose-response variable slope model in GraphPad Prism 5.

2.2. Glucose uptake in rat primary hepatocytes

Hepatocytes were isolated by *in situ* liver perfusion with collagenase. The viability of isolated hepatocytes were determined to be over 85% via Trypan blue exclusion assay. Cells were suspended in MEM supplemented with 10% fetal bovine serum (v/v), 100 U/mL penicillin, and 100 μg/mL streptomycin. 1×10^6 cells/well were planted onto collagen 1-coated 12-well plates. After 36 h incubation (37 ℃; 5% CO_2/95% air v/v) to allow cell attachment, the medium was replaced with serum-free media for a further 12 h culture. 2-Deoxy-D-[^{3}H]-glucose uptake was assessed in cultured hepatocytes. Cells were washed and incubated in serum-free MEM containing 5.5 mM glucose, and 2 μCi/mL 2-deoxy-D-[^{3}H]-glucose with or without the test compounds for 4 h. The reaction was terminated and washed three times with ice-cold phosphate buffered saline (PBS), and then cells were lysed with 0.1 N NaOH. Portions of cell lysates were used for scintillation counting, and the results were recorded as increased percentage versus control.

2.3. Insulin secretion in rat pancreatic islets

An insulin secretion assay was performed using pancreatic islets isolated from male Sprague-Dawley rats. Insulin release was determined during static islet incubation. Briefly, groups of five islets were placed in incubation wells. After a 30-min pre-incubation with HEPES-buffered Krebs-Ringer buffer (pH 7.4) containing 5 mm glucose, islets were transferred to wells containing 2 ml HEPES-buffered Krebs-Ringer buffer and varying concentrations of glucose and the test compounds. The studies were performed at 37 ^{o}C in a waterbath shaker with an atmosphere of 5% CO_2. Samples of incubation buffer were collected at 1 h for insulin determination using a mouse ELISA kit (Mercodia, Uppsala, Sweden). Results were recorded as the increased percentage versus control.

2.4. *In vivo* oral glucose tolerance test (OGTT) assay

Anti-diabetic effects of the compounds were evaluated by a glucose tolerance test (OGTT) using normal and diabetic db/db mice. Eight-week old male C57BL/6 mice (OrientBio Inc, Republic of Korea) were fasted overnight (with free access to water) before performing the test. The mice were orally administered with the compound or vehicle alone (0.5% methylcellulose solution). After 30 min, the mice were administered with an oral glucose challenge (2 g/kg). Blood glucose concentrations were measured by GlucoDr AMG-3000 (Allmedicus Inc., Republic of Korea) just prior to and following the glucose challenge (30, 60, 90, 120 and 180 min) from the tail tip. AUC values of the time-glucose curve were calculated from the data.

3. Pharmacokinetics (PK) protocol

ICR male mice received an intravenous bolus (3 mg/kg) and oral gavage doses (10 mg/kg) of the test compound. Blood samples were obtained at 0.08 (iv only), 0.25, 0.5, 1, 2, 4,

and 7 h after drug administration. Plasma samples were obtained following centrifugation of blood at 4 ℃ and stored at -20 ℃ for analysis. To a 0.1 mL aliquot of mouse plasma, 0.2 mL of acetonitrile was added. The mixture was vortexed for 10 min and then centrifuged for a further 10 min. The supernatant was then tested for test compound concentration via HPLC. Pharmacokinetic variables were evaluated by noncompartmental analysis using WinNonlin Professional Version 5.2 (Pharsight, MO, USA).