Discovery of MK-8722: A Systemic, Direct Pan-Activator of AMP-Activated Protein Kinase

Danqing Feng[1], Tesfaye Biftu[1], F. Anthony Romero[1], Ahmet Kekec[1], James Dropinski[1], Andrew Kassick[1], Shiyao Xu[2], Marc M. Kurtz[3], Anantha Gollapudi[4], Qing Shao[4], Xiaodong Yang[5], Ku Lu[5], Gaochao Zhou[5], Daniel Kemp[5], Robert W. Myers[5], Hong-Ping Guan[5], Maria E. Trujillo[4], Cai Li[5], Ann Weber[1] and Iyassu K. Sebhat[1]*

Supporting Information

Table S1. Activation of mouse AMPK complexes by compound 3a

AMPK Complex	Subunits	Mouse
AMPK1	α1β1γ1	1.4 (360%)
AMPK2	α1β1γ2	1.8 (465%)
AMPK3	α1β1γ3	2.5 (958%)
AMPK4	α1β2γ1	25 (546%)
AMPK5	α1β2γ2	22 (536%)
AMPK6	α1β2γ3	20 (511%)
AMPK7	α2β1γ1	0.93 (889%)
AMPK8	α2β1γ2	1.8 (1125%)
AMPK9	α2β1γ3	4.7 (1973%)
AMPK10	α2β2γ1	34 (1023%)
AMPK11	α2β2γ2	28 (856%)
AMPK12	α2β2γ3	19 (974%)

Experimental

Preparation of 5-((5-([1,1'-biphenyl]-4-yl)-6-chloro-1*H*-benzo[*d*]imidazol-2-yl)oxy)-2-methylbenzoic acid (3).

Step 1: 5-Chloro-4-iodo-2-nitroaniline. To a solution of 5-chloro-2-nitroaniline (145 mmol, 1 equiv) in AcOH (250 mL) was added *N*-iodosuccinimide (145 mmol, 1 equiv). The mixture was stirred overnight at 50 °C, cooled down to rt and filtered. The solid residue was washed with AcOH, water, saturated aqueous $NaHCO_3$ and water, and then dried to afford the desired product as a brown solid, which was used in the next step without further purification.

Step 2: 4-Chloro-5-iodobenzene-1,2-diamine. To a suspension of 5-Chloro-4-iodo-2-nitroaniline (120 mmol) in EtOH (800 mL) and water (150 mL) was added iron powder (670 mmol) and NH_4Cl (310 mmol). The mixture was heated under nitrogen at 50°C overnight. Additional iron powder (670 mmol) and NH_4Cl (310 mmol) were added and heating was continued for 45 h. The reaction mixture was cooled, filtered and concentrated. The residue was dissolved in ethyl acetate and washed with sodium bicarbonate solution. The organic phase was concentrated to afford the desired product as a gray solid, which was used in the next step without further purification.

Step 3: 5-Chloro-6-iodo-1,3-dihydro-2*H*-benzimidazole-2-thione. KOH (240 mmol) in water (50 mL), followed by carbon disulfide (240 mmol), was added to a solution of 4-Chloro-5-iodobenzene-1,2-diamine (200 mmol) in EtOH (300 mL). The mixture was heated at reflux for 3 h, cooled and filtered. To the filtrate was added water (300 mL) and then AcOH (25 mL) in water (50 mL). The precipitate was collected, washed with water and a small amount of EtOH and dried to afford the desired product as a brown powder, which was used in the next step without further purification.

Step 4: 6-Chloro-5-iodo-2-(methylthio)-1*H*-benzimidazole. K_2CO_3 (1.6 mmol), followed by iodomethane (1.6 mmol), was added to a solution of 5-Chloro-6-iodo-1,3-dihydro-2*H*-benzimidazole-2-thione (3.2 mmol) in acetone (20 mL) at 0°C. The reaction was stirred at rt for 1 h. Additional K_2CO_3 (1.6 mmol) and iodomethane (1.6 mmol) were added, and stirring continued at rt overnight. Volatiles were removed and the residue was partitioned between EtOAc and water. Concentration afforded the desired product as a white foam, which was used in the next step without further purification.

Step 5: 6-chloro-5-iodo-2-(methylsulfonyl)-1H-benzo[*d*]imidazole. *m*-Chloroperbenzoic acid (6.2 mmol) was added to a suspension of 6-chloro-5-iodo-2-(methylthio)-1*H*-benzimidazole (3.1 mmol) in DCM (50 mL). The reaction stirred at rt for 10 min then washed with 10% aqueous $NaHCO_3$. The organic phase was concentrated. The residue was triturated with MeOH (3 mL) and filtered to afford the title compound as white powder. LC-MS: calculated for $C_8H_6ClIN_2O_2S$ 356.90, observed m/e 357.30 $(M + H)^+$. ^1H NMR (500 MHz, CD_3OD) δ 8.3 (1H, s), 7.9 (1H, s), 3.3 (3H, s).

Step 6: 5-((5-([1,1'-biphenyl]-4-yl)-6-chloro-1*H*-benzo[*d*]imidazol-2-yl)oxy)-2-methylbenzoic acid. A solution of potassium phosphate, tribasic (2 M solution in water) (130 mmol), $Pd(PPh_3)_4$ (1.7 mmol), 4-biphenylboronic acid (63 mmol) and 6-chloro-5-iodo-2-(methylsulfonyl)-1H-benzo[*d*]imidazole (42 mmol) in dioxane (300 mL) was heated at 100°C for 5 h. The aqueous phase was removed and the organic phase was concentrated, diluted with EtOAc and DCM, filtered and concentrated to afford a white solid. The white solid was then mixed with methyl 5-hydroxy-2-methylbenzoate (18 mmol) in DCM (100 mL), and the mixture was concentrated. The resulting solid was placed under nitrogen in a sealed vessel and heated at 130°C for 3 h, then cooled and mixed with EtOAc and water. The mixture was filtered and the organic phase was concentrated. Chromatography over silica eluting with 40% EtOAc/hexane afforded a white foam. A solution of this product in MeOH/water (1:1) (250 mL) was mixed with NaOH (5 M in water) (23 mL, 120 mmol). The mixture was heated at 70 °C for 1 h. The mixture was then cooled, diluted with water and acidified with 2 M aqueous HCl. The precipitated white solid was filtered and dried to afford the title compound as a white powder. LCMS: calculated for $C_{27}H_{20}ClN_2O_3$ 455.11, observed m/e 455.5 (M+H) (Rt 2.27/4min) ^1H NMR (500 MHz, Acetone-d6) δ 8.00 (1 H, d, *J* = 2.74 Hz), 7.74-7.71 (4 H, m), 7.60-7.58 (2 H, m), 7.55-7.53 (2 H, m), 7.49-7.46 (3 H, m), 7.42 (1 H, d, *J* = 8.41 Hz), 7.38-7.35 (1 H, m), 2.60 (3 H, s).

Preparation of cis-4-{[5-(biphenyl-4-yl)-4,6-difluoro-1H-benzimidazol-2-yl]oxy}-cyclohexane carboxylic acid (4).

Step 1: N-(biphenyl-4-ylmethyl)-3,5-difluoro-2-nitroaniline. Potassium carbonate (11 g, 79 mmol) was added to a solution of 1,3,5-trifluoro-2-nitrobenzene and 1-biphenyl-4-ylmethanamine in THF (200 mL). The mixture was stirred at room temperature for 15 h. Then the reaction mixture was filtered and concentrated to afford the desired product as a deep orange solid which was used in subsequent steps without further purification.

Step 2: N-(biphenyl-4-ylmethyl)-3,5-difluoro-4-iodo-2-nitroaniline. NIS (7.9 g, 35 mmol) was added to a solution of N-(biphenyl-4-ylmethyl)-3,5-difluoro-2-nitroaniline (11 g, 32 mmol) in AcOH (150 mL). After heating at 70°C for 2 h, the reaction mixture was concentrated and partitioned between EtOAc and saturated aqueous $NaHCO_3$. The organic phase was separated, washed with brine, dried (Na_2SO_3) and concentrated. The resulting solid was recrystallized from DCM/hexanes to afford the desired product as a red solid which was used in subsequent steps without further purification.

Step 3: N'-(biphenyl-4-ylmethyl)-3,5-difluoro-4-iodobenzene-1,2-diamine. A 20% solution of AcOH (6.7 mL, 120 mmol) in water was added to a suspension of iron (11 g, 195 mmol) in a solution of N-(biphenyl-4-ylmethyl)-3,5-difluoro-4-iodo-2-nitroaniline (12 g, 26 mmol) in EtOH (70 mL). After heating the reaction at 76 °C for 2 h, the volatiles were removed. The resulting residue was extracted with EtOAc. The combined organic extracts were filtered through Celite™, washed with aqueous ammonium hydroxide and brine, dried (Na_2SO_4) and concentrated. Chromatography of the resulting residue over silica eluting with 10-50% EtOAc/hexanes afforded the desired product as a yellow solid.

Step 4: 1-(biphenyl-4-ylmethyl)-4,6-difluoro-5-iodo-1,3-dihydro-2H-benzimidazole-2-thione. 1,1'-Thiocarbonyldiimidazole (4.8 g, 27 mmol) was added to a solution of N-(biphenyl-4-ylmethyl)-3,5-difluoro-4-iodobenzene-1,2-diamine (9.7 g, 22 mmol) in DMSO (30 mL). After stirring at rt for 16 h, the reaction mixture was diluted with DCM and the precipitated solid was collected to afford the desired product which was used in subsequent steps without further purification.

Step 5: 1-(biphenyl-4-ylmethyl)-4,6-difluoro-5-iodo-2-(methylthio)-1H-benzimidazole. Iodomethane (2M in methyl-tert-butyl ether, 23 mL, 46 mmol) was added to a solution of cesium carbonate (15 g, 46 mmol) and 1-(biphenyl-4-ylmethyl)-4,6-difluoro-5-iodo-1,3-dihydro-2H-benzimidazole-2-thione (11 g, 23 mmol) in THF (100 mL). After stirring the reaction at rt overnight, the volatiles were removed. Chromatography of the resulting residue over silica eluting with 15-60% EtOAc/hexanes afforded the desired product as a beige solid.

Step 6 (**intermediate 1**): 1-(biphenyl-4-ylmethyl)-4.6-difluoro-5-iodo-2-(methylsulfonyl)-1H-benzimidazole. m-CPBA (10 g, 45 mmol) in DCM (200 mL) was added to 1-(biphenyl-4-ylmethyl)-4,6-difluoro-5-iodo-2-(methylthio)-1H-benzimidazole (10.98 g, 22.3 mmol). The reaction mixture was stirred at rt for 16 h. An additional portion of m-CPBA (3 g) was added and the reaction was stirred for 1 h. The volatiles were removed, and the resulting residue was partitioned between EtOAc and saturated aqueous $NaHCO_3$. The organic phase was separated, washed with brine, dried (Na_2SO_4) and concentrated. Chromatography of the resulting residue over silica eluting with 15-30% EtOAc/hexanes afforded the title compound as a white solid. LC-MS: calculated for $C_{21}H_{16}F_2IN_2O_2S$ 524.99, observed m/e 525.00 (M + H)+ (Rt 2.15/4 min).

Step 7 (**intermediate 2**): ethyl 4-{[1-(biphenyl-4-ylmethyl)-4,6-difluoro-5-iodo-1H-benzimidazol-2-yl]oxy}cyclohexanecarboxylate. To a solution of 1-(biphenyl-4-ylmethyl)-4,6-difluoro-5-iodo-2-(methylsulfonyl)-1H-benzimidazole (intermediate 1, 20 g, 38 mmol) and ethyl 4-hydroxycyclohexanecarboxylate (21 ml, 130 mmol, Aldrich, a mixture of cis and trans isomers) in DMF (70 mL) was added DBU (23 mL, 130 mmol) dropwise at room temperature. The reaction mixture was heated at 80 °C overnight. Then the volatiles were removed in vacuo and the resulting residue partitioned between EtOAc and water. The organic phase was separated, washed with water and brine, dried over $MgSO_4$ and concentrated in vacuo. Chromatography of the resulting residue over silica eluting with 15% EtOAc/hexane afforded the title compound as a mixture of trans and cis isomers. LC-MS: calculated for $C_{29}H_{28}F_2IN_2O_3$ 617.11, observed m/e: 617.20 (M+H) + (Rt 2.82/4 min).

Step 8: ethyl 4-{[5-(biphenyl-4-yl)-1-(biphenyl-4-ylmethyl)-4,6-difluoro-1H-benzimidazol-2-yl]oxy}cyclohexanecarboxylate. A 250 mL flask was charged with ethyl 4-{[1-(biphenyl-4-ylmethyl)-4,6-difluoro-5-iodo-1 H-benzimidazol-2-yl] oxy}cyclohexanecarboxylate (intermediate 2, 2 g, 3.2 mmol), 4-biphenylboronic acid (0.70 g, 3.5 mmol), Pd(PPh3)4 (0.19 g, 0.16 mmol), DMF (30 mL), and 1 M aqueous K_2CO_3 (6.5 mL, 6.5 mmol). The reaction was degassed with N_2 and then heated at 120 °C for 1 h. The volatiles were removed in vacuo and the resulting residue was partitioned between EtOAc and water. The aqueous phase was extracted with EtOAc. The combined organic layers were washed with brine, dried ($MgSO_4$), filtered, and concentrated. Chromatography of the resulting residue over silica eluting

with 15% [EtOAc:DCM (1:1)]/hexanes afforded the desired trans product as white solid. LC-MS: calculated for C41H36F2N203 642.27, observed m/e: 643.14 (M+H) + (Rt 3.07/4 min).

Step 9: Ethyl 4-{[5-(biphenyl-4-yl)-4,6-difluoro-1H-benzimidazol-2-yl]oxy}cyclohexanecarboxylate. A suspension of ethyl 4-{[5-(biphenyl-4-yl)-1-(biphenyl-4-ylmethyl)-4,6-difluoro-1H-benzimidazol-2-yl]oxy}cyclohexane carboxylate (1.1 g, 1.6 mmol) and Pearlman's Catalyst (0.30 g, 0.43 mmol) in ethyl acetate (15 mL) and EtOH (7.5 mL) was treated with 1,4-cyclohexadiene (3.6 mL, 3.8 mmol). The resulting reaction mixture was microwaved at 120 °C for 90 min, and then cooled. The cooled reaction mixture was filtered through a Celite™ pad and the volatiles were removed in vacuo. Chromatography of the resulting residue over silica eluting with 20% THF/hexanes afforded the desired trans product as white solid. LC-MS: calculated for $C_{28}H_{27}F_2N_2O_3$ 477.20, observed m/e: 477.01 (M+H) + (Rt 2.6/4 min).

Step 10: cis-4-{[5-(biphenyl-4-yl)-4,6-difluoro-1H-benzimidazol-2-yl]oxy}-cyclohexane carboxylic acid. To a solution of ethyl 4-{[5-(biphenyl-4-yl)-4,6-difluoro-1H-benzimidazol-2-yl]oxy}cyclohexanecarboxylate (0.62 g, 1.3 mmol) in THF (5 mL) was added potassium trimethyl silanolate (0.55 g, 4.3 mmol). The reaction was stirred overnight at ambient temperature. Then the volatiles were removed in vacuo and the resulting residue was acidified with 1N aqueous HCI and extracted with EtOAc. The organic phase was washed with water and concentrated in vacuo. Purification of the resulting residue by reverse phase HPLC eluting with 50-100% MeCN: H_2O afforded the title compound as a white solid. LC-MS: calculated for $C_{26}H_{23}F_2N_2O_3$ 449.17, observed m/e 449.01 (M + Ht (Rt 2.32/4 min). 1H NMR (500 MHz, DMSO-d_6): δ 7.80-7.70 (m, 4H), 7.55-7.45 (m, 4H), 7.40-7.35(m, 1H), 7.20-7.15 (m, 1H), 5.00-4.90 (m, 1H), 2.35-2.20 (m, 3H), 2.05-1.95 (m, 2H), 1.60-1.50 (m, 4H).

Preparation of cis-4-((4,6-difluoro-5-(4-(pyrrolidin-1-yl)phenyl)-1H-benzo[d]imidazol-2-yl)oxy)cyclohexane-1-carboxylic acid (4a).

Step 1: Ethyl 4-((1-([1,1'-biphenyl]-4-ylmethyl)-4,6-difluoro-5-(4-(pyrrolidin-1-yl)phenyl)-1H-benzo[d]imidazol-2-yl)oxy)cyclohexane-1-carboxylate . A 50 mL flask was charged with ethyl 4-{[1-(biphenyl-4-ylmethyl)-4,6-difluoro-5-iodo-1 H-benzimidazol-2-yl] oxy} cyclohexane carboxylate (intermediate 2, 0.2 g, 0.30 mmol) , pyrrolidino boronate ester (0.10 g, 0.36 mmol), Pd(PPh3)4 (0.015 g, 0.013 mmol), DMF (3.2 mL), and 1 M aqueous K_2CO_3 (0.65 ml, 0.65mmol). The reaction was degassed with N_2 and then heated at 1100C for 40 minutes. The volatiles were removed in vacuo and the resulting residue was partitioned between EtOAc and H20. The aqueous phase was extracted with EtOAc. The combined organic layers were washed with brine, dried ($MgSO_4$), filtered, and concentrated. Chromatography of the resulting residue over silica eluting with 20% [EtOAc/Hexanes] afforded the desired trans product as white solid. LC-MS: calculated for C39H40F2N3O3 636.30, observed m/e: 636.00 (M+H) + (Rt 2.65/4 min).

Step 2: Ethyl 4-((4,6-difluoro-5-(4-(pyrrolidin-1-yl)phenyl)-1H-benzo[d]imidazol-2-yl)oxy)cyclohexane-1-carboxylate. A suspension of Ethyl 4-((1-([1,1'-biphenyl]-4-ylmethyl)-4,6-difluoro-5-(4-(pyrrolidin-1-yl)phenyl)-1H-benzo[d]imidazol-2-yl)oxy)cyclohexane-1-carboxylate (0.15 g, 0.24 mmol) and Pearlman's Catalyst (0.043 g, 0.061 mmol) in ethyl acetate (2.3 mL) and EtOH (1.2 mL) was treated with 1 ,4-cyclohexadiene (0.57 ml, 6.1 mmol). The resulting reaction mixture was microwaved at 130 °C for 120 min, and then cooled. The cooled reaction mixture was filtered through a Celite™ pad and the volatiles were removed in vacuo. Crude product was isolated as a white solid and used in the next step without any further purification. LC-MS: calculated for $C_{26}H_{30}F_2N_3O_3$ 470.23, observed m/e: 469.99 (M+H) + (Rt 2.03/4 min).

Step 3: 4-((4,6-difluoro-5-(4-(pyrrolidin-1-yl)phenyl)-1H-benzo[d]imidazol-2-yl)oxy)cyclohexane-1-carboxylic acid. To a solution of ethyl 4-((4,6-difluoro-5-(4-(pyrrolidin-1-yl)phenyl)-1H-benzo[d]imidazol-2-yl)oxy)cyclohexane-1-carboxylate (0.11 g, 0.24 mmol) in THF (1.6 mL) was added potassium trimethyl silanolate (0.12 g, 0.95 mmol). The reaction was stirred overnight at ambient temperature. Then the volatiles were removed in vacuo and the resulting residue was acidified with 1N aqueous HCI and extracted with EtOAc. The organic phase was washed with water and concentrated in vacuo. Purification of the resulting residue by reverse phase HPLC eluting with 10-100% MeCN:H_2O afforded the title compound as a white solid. LC-MS: calculated for $C_{24}H_{26}F_2N_3O_3$ 442.19, observed m/e 442.24 (M + Ht (Rt 1.87 /4 min). 1H NMR (500 MHz, DMSO-d_6): δ 7.22-7.18 (m, 2H), 7.06-7.02 (m, 1H), 6.64-6.60 (m, 2H), 4.96-4.88 (m, 1H), 3.28-3.22 (m, 4H), 2.30-2.16(m, 3H), 2.00-1.92 (m, 6H), 1.54-1.46 (m, 4H).

Preparation of cis-4-((6-chloro-5-(4-(pyrrolidin-1-yl)phenyl)-1H-imidazo[4,5-b]pyridin-2-yl)oxy)cyclohexane-1-carboxylic acid (4b).

Step 1: 5, 6-dichloro-3-nitropyridin-2-amine: To a solution of 5-chloro-3-nitropyridin-2-amine (16 g, 92 mmol) in AcOH (70 mL) was added N-chlorosuccinimide (15 g, 110 mmol). The mixture was stirred overnight at 80 °C for 3 h, cooled to rt, diluted with MeOH (30 mL) and filtered. The solid residue was washed with AcOH, water, and then dried to afford the desired product as a white solid (15 g, 72 mmol, 78%), which was used in the next step without further purification. LC-MS: calculated for $C_5H_4Cl_2N_3O_2$ 207.97, observed m/e: 208.07 $(M+H)^+$ (Rt 1.48/5 min).

Step 2: 5-chloro-6-iodo-3-nitropyridin-2-amine. To a solution of 5,6-dichloro-3-nitropyridin-2-amine (15 g, 72 mmol) in AcOH (70 mL) was added sodium iodide (43 g 150 mmol). The mixture was stirred at 90 °C for 2 h, cooled to rt, diluted with water (70 mL) and filtered. The solid residue was washed with water, and then dried under vacuum to afford the desired product as a pale yellow solid (19 g, 63 mmol, 88%), which was used in the next step without further purification. LC-MS: calculated for $C_5H_4ClIN_3O_2$ 299.90, observed m/e: 299.94 $(M+H)^+$ (Rt 2.18/5 min).

Step 3: 5-chloro-6-iodopyridine-2,3-diamine. To a suspension of 5-chloro-6-iodo-3-nitropyridin-2-amine (18.9 g, 63.1 mmol) in EtOH (100 mL) was added tin (II) chloride dihydrate (57 g, 252 mmol). The mixture was heated at 70 °C for 0.5 h. The rxn was warmed to rt and treated with a slurry of 150 mL water and 60 g KF and stirred for 0.5 h. The mixture was then partitioned between ethyl acetate (300 mL) and water (300 mL). The ethyl acetate layer was washed with brine, dried over magnesium sulfate and filtered through a 100 g pad of silica gel. The filtrate was concentrated and dried under vacuum to give an off-white solid (17 g, 63 mmol, 100%), which was used in next step without further purification. LC-MS: calculated for $C_5H_6ClIN_3$ 269.93, observed m/e: 269.99 $(M+H)^+$ (Rt 1.35/5 min).

Step 4: 6-chloro-5-iodo-1,3-dihydro-2H-imidazo [4,5-b] pyridine-2-thione. DMAP (15.4 g, 126 mmol) was added to a THF (200 mL) solution of 5-chloro-6-iodopyridine-2,3-diamine (17 g, 63.1 mmol). Thiophosgene (4.9 mL, 63.1 mmol) was then added drop-wise via addition funnel under nitrogen and allowed to stir at rt for 1 h. The mixture was then partitioned between ethyl acetate (500 mL) and 2N HCl (100 mL). The ethyl acetate layer was washed with brine, dried over magnesium sulfate and concentrated to give the desired product as a white powder (11g, 35.3 mmol, 56%), which was used in the next step without further purification. LC-MS: calculated for $C_6H_4ClIN_3S$ 311.89, observed m/e: 311.91 $(M+H)^+$ (Rt 1.69/5 min).

Step 5: 6-chloro-5-iodo-2-(methysulfanyl)-1H-imidazo[4,5-b] pyridine. A suspension of 6-chloro-5-iodo-1,3-dihydro-2H-imidazo [4,5-b] pyridine-2-thione (11 g, 35 mmol) and KOH (2.4 g, 42 mmol) in ethanol (200 mL) was stirred at rt for 0.5 h. Iodomethane (2.2 mL, 35 mmol) was then added and the reaction was allowed to stir for 1 h at rt. The ethanol was removed in vacuo and the resulting residue was partitioned between ethyl acetate (250 mL) and 2N HCl (50 mL). The ethyl acetate layer was washed with brine, dried over magnesium sulfate, filtered through a 100 g pad of silica gel and concentrated to give the desired product as a white solid (5.0g, 15 mmol, 44%) LC-MS: calculated for $C_7H_6ClIN_3S$ 325.90, observed m/e: 325.88 $(M+H)^+$ (Rt 2.05/5 min).

Step 6 (intermediate 3): 6-chloro-5-iodo-2-(methsulfonyl)-1H-imidazo[4,5-b] pyridine. Oxone (20.8 g, 33.8 mmol) was added to an acetonitrile (100 mL)/water (100 mL) suspension of 6-chloro-5-iodo-2-(methysulfanyl)-1H-imidazo[4,5-b] pyridine (5.0 g, 15.4 mmol) and the reaction was allowed to stir for 18 h at rt. The suspension was filtered through a sintered glass funnel and the filtrate was partitioned between ethyl acetate and saturated sodium bisulfate. The ethyl acetate layer was washed with brine, dried over magnesium sulfate and concentrated to afford the title compound as a white solid (4.7g, 13.2 mmol, 86%) that was used in subsequent steps without further purification. Solubility precludes purification and this was used as is. LC-MS: calculated for $C_7H_5ClIN_3O_2S$ 357.89, observed m/e: 357.07 $(M+H)^+$ (Rt 1.36/4 min) ^1H NMR δ (ppm)(DMSO-d_6): 8.44 (1 H, s), 3.53 (3 H, s).

Step 7: Ethyl 4-((1-allyl-6-chloro-5-iodo-1H-imidazo[4,5-b]pyridin-2-yl)oxy)cyclohexane-1-carboxylate. Sodium hydride (480 mg, 20 mmol) was added to a DMF solution of 6-chloro-5-iodo-2-(methsulfonyl)-1H-imidazo[4,5-b] pyridine (5.9 g, 17 mmol) and allyl bromide (1.7 mL, 20 mmol) at rt. The reaction was allowed to stir at rt for 16 h. The reaction was then treated sequentially with cis- and trans-ethyl-4-hydroxycyclohexanecarboxylate (11 mL, 66 mmol) and DBU (10 mL, 66 mmol). The reaction was partitioned between EtOAc and 10% aqueous citric acid. The EtOAc layer was washed with water, brine, dried over magnesium sulfate and concentrated. Flash chromatography of the resulting residue utilizing a Biotage™ 100G SNAP cartridge and employing a gradient: 0-20% EtOAc/hexane afforded the title compound as a colorless oil, which solidified after overnight vacuum drying (1.6g, 3.3 mmol, 20%). LC-MS: calculated for $C_{18}H_{22}ClIN_3O_3$ 490.04, observed : m/e: 489.9 $(M+H)^+$ (Rt 2.75/4 min).

Step 8: Ethyl cis-4-((1-allyl-6-chloro-5-(4-(pyrrolidin-1-yl)phenyl)-1H-imidazo[4,5-b]pyridin-2-yl)oxy)cyclohexane-1-carboxylate. 1,1'-bis(diphenylphosphinoferrocene-Palladium (II) dichloride dichloromethane complex (434 mg, 0.53 mmol) was added to a 9:1 dioxane/water (10 mL) solution of ethyl 4-((1-allyl-6-chloro-5-iodo-1H-imidazo[4,5-b]pyridin-2-yl)oxy)cyclohexane-1-carboxylate. (1.3 g, 2.7 mmol), 1-[4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl]pyrrolidine (940 mg, 3.5 mmol) and lithium hydroxide (190 mg, 8.0 mmol) at rt under a nitrogen atmosphere. The reaction was heated to 90 °C for 3 h. The reaction was cooled and partitioned between EtOAc and 10% aqueous citric acid. The organic layer was washed with water and brine, dried over magnesium sulfate and concentrated. Flash chromatography of the resulting residue utilizing a Biotage™ 50G SNAP cartridge and employing a linear gradient: 0-100% EtOAc/hexane afforded the title compound as a white solid (500 mg, 0.98 mmol, 40%). LC-MS: calculated for $C_{28}H_{34}ClN_4O_3$ 509.23, observed m/e: 509.21 $(M+H)^+$ (Rt 1.5/2 min).

Step 9: Ethyl cis-4-((6-chloro-5-(4-(pyrrolidin-1-yl)phenyl)-1H-imidazo[4,5-b]pyridin-2-yl)oxy)cyclohexane-1-carboxylate. Tetrakis(triphenylphosphine)palladium(0) (110 mg, 0.1 mmol) was added to a DMA (5 mL) solution of dimethylbarbituric acid (310 mg, 2.0 mmol) and ethyl cis-4-((1-allyl-6-chloro-5-(4-(pyrrolidin-1-yl)phenyl)-1H-imidazo[4,5-b]pyridin-2-yl)oxy)cyclohexane-1-carboxylate (500 mg, 0.98 mmol) at rt under a nitrogen atmosphere. The reaction was heated to 75 °C for 16 h. The reaction was cooled and partitioned between EtOAc and 10% aqueous citric acid. The organic layer was washed with water and brine, dried over magnesium sulfate and concentrated. Flash chromatography of the resulting residue utilizing a Biotage™ 50G SNAP cartridge and employing a linear gradient: 0-100% EtOAc/hexane afforded the title compound as a white solid (220 mg, 0.47 mmol, 48%). LC-MS: calculated for $C_{25}H_{30}ClN_4O_3$ 469.20, observed m/e: 469.9 $(M+H)^+$ (Rt 1.4/2 min).

Step 10: cis-4-((6-chloro-5-(4-(pyrrolidin-1-yl)phenyl)-1H-imidazo[4,5-b]pyridin-2-yl)oxy)cyclohexane-1-carboxylic acid. Potassium trimethylsilanolate (744mg, 5.8 mmol) was added to a THF (10 mL) solution of ethyl cis-4-((6-chloro-5-(4-(pyrrolidin-1-yl)phenyl)-1H-imidazo[4,5-b]pyridin-2-yl)oxy)cyclohexane-1-carboxylate (680 mg, 1.5 mmol), and the reaction was stirred at rt for 16 h. The reaction mixture was then partitioned between EtOAc and 10% aqueous citric acid. The organic layer was washed with brine, dried over magnesium sulfate and concentrated rinsing with THF (40 mL). The combined organic layers were concentrated, and then triturated with acetonitrile (50 mL) to afford the title compound as an off-white solid (220 mg, 0.5 mmol, 34%). LC-MS: calculated for $C_{23}H_{26}ClN_4O_3$ 441.17, observed m/e: 441.1 $(M+H)^+$ (Rt 1.9/4 min).

Preparation of (2R,4S)-4-((5-([1,1'-biphenyl]-4-yl)-4,6-difluoro-1H-benzo[d]imidazol-2-yl)oxy)tetrahydro-2H-pyran-2-carboxylic acid (5).

Step 1: ethyl 4-((1-([1,1'-biphenyl]-4-ylmethyl)-4,6-difluoro-5-iodo-1H-benzo[d]imidazol-2-yl)oxy)tetrahydro-2H-pyran-2-carboxylate. To a solution of 1-(biphenyl-4-ylmethyl)-4,6-difluoro-5-iodo-2-(methylsulfonyl)-1H-benzimidazole (intermediate 1, 0.7 g, 1.3 mmol) and ethyl 4-hydroxytetrahydro-2H-pyran-2-carboxylate (0.58 mg, 3.3 mmol) in DMF (6.7 ml) was added Cs2CO3 (1.0 g, 3.1 mmol) portionwise at room temperature. The reaction mixture was heated at 35 °C overnight. Then the volatiles were removed in vacuo and the resulting residue partitioned between EtOAc and water. The organic phase was separated, washed with water and brine, dried over $MgSO_4$ and concentrated in vacuo. Chromatography of the resulting residue over silica eluting with 15% EtOAc/hexane afforded the title compound. LC-MS: calculated for $C_{28}H_{26}F_2IN_2O_4$ 619.09, observed m/e: 618.90 $(M+H)^+$ (Rt 2.45/4 min).

Step 2: ethyl 4-((5-([1,1'-biphenyl]-4-yl)-1-([1,1'-biphenyl]-4-ylmethyl)-4,6-difluoro-1H-benzo[d]imidazol-2-yl)oxy)tetrahydro-2H-pyran-2-carboxylate. A 50 mL flask was charged with Ethyl 4-((1-([1,1'-biphenyl]-4-ylmethyl)-4,6-difluoro-5-iodo-1H-benzo[d]imidazol-2-yl)oxy)tetrahydro-2H-pyran-2-carboxylate (0.21 g, 0.35 mmol), 4-Biphenylboronic acid (0.075 g, 0.38 mmol), Pd(PPh3)4 (0.02 g, 0.017 mmol), DMF (3.5 mL), and 1 M aqueous K2CO3 (0.69 ml, 0.69 mmol). The reaction was degassed with N2 and then heated at 120 °C for 30 min. The volatiles were removed in vacuo and the resulting residue was partitioned between EtOAc and water. The aqueous phase was extracted with EtOAc. The combined organic layers were washed with brine, dried ($MgSO_4$), filtered, and concentrated. Chromatography of the resulting residue over silica eluting with 30% [EtOAc/Hexanes] afforded the desired product as a white solid. LC-MS: calculated for $C_{40}H_{35}F_2N_2O_4$ 645.26, observed m/e: 645.10 $(M+H)^+$ (Rt 2.71/4 min).

Step 3: ethyl 4-((5-([1,1'-biphenyl]-4-yl)-4,6-difluoro-1H-benzo[d]imidazol-2-yl)oxy)tetrahydro-2H-pyran-2-carboxylate. A solution of ethyl 4-((5-([1,1'-biphenyl]-4-yl)-1-([1,1'-biphenyl]-4-ylmethyl)-4,6-difluoro-1H-benzo[d]imidazol-2-yl)oxy)tetrahydro-2H-pyran-2-carboxylate (0.1 g, 0.16 mmol) and Pearlman's Catalyst (0.028 g, 0.04 mmol) in ethyl acetate (1.5 mL) and EtOH (0.75 mL) was treated with 1 ,4-cyclohexadiene (0.37 ml, 4.0 mmol). The resulting reaction mixture was microwaved at 130 °C for 120 min, and then cooled. The cooled reaction mixture was filtered through a Celite™ pad and the volatiles were removed in vacuo. Chromatography of the resulting residue over silica eluting with 45% EtOAc/hexanes afforded the desired trans product as a white solid. LC-MS: calculated for $C_{27}H_{25}F_2N_2O_4$ 479.18, observed m/e: 479.00 (M+H) + (Rt 2.21/4 min).

Step 4: (2R,4S)-4-((5-([1,1'-biphenyl]-4-yl)-4,6-difluoro-1H-benzo[d]imidazol-2-yl)oxy)tetrahydro-2H-pyran-2-carboxylic acid. To a solution of ethyl 4-((5-([1,1'-biphenyl]-4-yl)-4,6-difluoro-1H-benzo[d]imidazol-2-yl)oxy)tetrahydro-2H-pyran-2-carboxylate (0.06 g, 0.13 mmol) in THF (1.5 mL) was added potassium trimethyl silanolate (0.048 g, 0.38 mmol). The reaction was stirred overnight at ambient temperature. Then the volatiles were removed in vacuo and the resulting residue was acidified with 1N aqueous HCI and extracted with EtOAc. The organic phase was washed with water and concentrated in vacuo. Purification of the resulting residue by reverse phase HPLC eluting with 10-100% MeCN: H2O afforded the title compound as a white solid. LC-MS: calculated for $C_{25}H_{21}F_2N_2O_4$ 451.15, observed m/e 451.00 (M + Ht (Rt 2.03 /4 min). 1H NMR (500 MHz, CD3OD): δ 7.72-7.66 (m, 4H), 7.54-7.50 (m, 2H), 7.47-7.43 (m, 2H), 7.37-7.32 (m, 1H), 7.04-7.00 (m, 1H), 5.25-5.15 (m, 1H), 4.20-4.15 (m, 2H), 3.70-3.60 (m, 1H), 2.70-2.60 (m, 1H), 2.30-2.20 (m, 1H), 1.85-1.75 (m, 2H).

Biological Study Procedures:

AMPK activity assay and determination of activator EC50 values

The enzymatic reaction was performed in three phases. In phase 1, the AMPK complex of interest was appropriately diluted (to twice the desired concentration in the final enzymatic reaction) in AMPK reaction buffer containing 20 mM HEPES, pH 7.3, 10 mM MgCl2, 6 mM DTT, 0.01% Brij 35, 200 μM ATP, and then phosphorylated/activated by addition of GST-tagged, truncated CaMKK2 (~20 nM final) (30) and incubation at room temperature for 30 min to yield pAMPK. For γ3 containing AMPK enzymes, the buffer used was 100mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM MgCl2, 6 mM DTT, 0.02% BSA and 200 uM ATP. In phase two, compound and pAMPK were pre-incubated by adding appropriately diluted AMPK activator (e.g., MK-8722 or AMP) in DMSO (1.2 μL total) to the reaction buffer containing pAMPK (15 μL per well; 384 well plates), the plate was vortexed briefly and then incubated at room temperature for 30 min. Finally, pAMPK reaction phase 3 was initiated by the addition of a fluorescein-labeled SAMS peptide, 5-FAM-HMRSAMSGLHLVKRR-COOH (ProfilerPro peptide 7, Perkin Elmer), in AMPK reaction buffer lacking ATP (15 μL; 1.5 μM final concentration peptide substrate). The plate was sealed and incubated at room temperature for 60 min, at which time the reaction was stopped by the addition of quench buffer consisting of 100 mM HEPES, pH 7.3, 0.015% Brij 35 and 40 mM EDTA. The reaction plate was centrifuged (2000 rpm for 4 min) and the supernatant read on the LabChip EZ reader (Perkin Elmer) using the following parameters: -550 downstream voltage, -2250 upstream voltage, -1.5 psi pressure, 40 s post-sample sip time, and 110 s final delay. The %product formed was calculated by taking the ratio of the substrate and product peak heights and multiplying by 100. EC50 and %activation parameters were calculated from %product vs. activator concentration plots using 4-parameter logistic curve fitting analysis.

Cell permeability

Parallel artificial membrane permeability (PAMPA) assay kit was obtained from pION (Billerica, MA). The initial donor concentration of tested compounds was 10 uM prepared in pION buffer. Acceptor buffer in the PAMPA kit was added to the acceptor. The assembled PAMPA system was incubated at room temperature for 4 hours. Acceptor and donor samples were collected and analyzed by LC-MS/MS. Effective permeability constant, Pe, was calculated according to the instruction manual for PSR4p permeability analyzer, rev 1.4.4. pION INC.

Plasma protein binding

Plasma protein binding to C57BL/6 mouse, Wistar-Han rat, rhesus macaque monkey and human plasma was determined by using a 96-well RED Equilibrium Dialyzer Plate (Harvard Apparatus, Holliston, MA). Equilibrium dialysis of plasma spiked with MK-8722 (10 μM) was performed against isotonic phosphate buffer (PBS). As MK-8722 is highly protein bound, diluted (10%) plasma was also included to estimate the binding of MK-8722 in undiluted (100%) plasma. At the end of the dialysis (4-6 hr), aliquots of the plasma and phosphate buffer were analyzed by LC-MS/MS to quantitate the MK-8722 in each compartment following protein precipitation using acetonitrile (see below). The unbound fraction of drug in plasma is calculated as follows:

% Unbound = (Concentration in buffer)/(Concentration in plasma) x 100%.

The unbound fraction in 10% plasma was also used to calculate the unbound fraction in undiluted (100%) plasma by taking the dilution factor (10x) into consideration. Both approaches yielded similar unbound fraction values in undiluted plasma.

OATP Uptake

The uptake of the test compound into MDCKII cells and MDCKII cells transiently transfected with OATP1B1 (MDCKII-OATP1B1) or OATP1B3 (MDCKII-OATP1B3) was measured. Briefly, twenty-four hours prior to the experiment, cells were treated with sodium butyrate (Sigma-Aldrich, St. Louis, MO) to increase expression of OATP1B1 or OATP1B3. Cells were dislodged with trypsin EDTA (Invitrogen) and re-suspended in Hanks' Balanced Salt Solution (HBSS) with 10 mM HEPES, pH 7.4. Uptake was initiated by the addition of radiolabeled test compound or positive control substrate [3H]estradiol-17β-glucuronide (E217βG; 1 μM) for OATP1B1 or positive control substrate [3H]cholecystokinin octapeptide (CCK8; 5 nM) for OATP1B3, with and without sulfobromophthalein sodium hydrate (BSP), a known OATP1B inhibitor. Cells were then incubated for the indicated time at 37°C in a temperature controlled shaker and uptake was stopped by the addition of ice cold PBS, immediate centrifugation for 1 minute at 3000 rpm at 4°C (Eppendorf, Model 5180R), followed by washing of the cell pellets with PBS, 3 times. Cell pellets were lysed in 50% acetonitrile (Sigma-Aldrich) and scintillation fluid was added (UltimaGold; Perkin Elmer, Waltham, MA). Radioactivity was determined by liquid scintillation counting in a MicroBeta Wallac Trilux scintillation counter (Perkin Elmer, Boston, MA). The experiment was performed in triplicate.

Animal Studies

All studies were conducted under the guiding principles of the American Physiological Society and the Guide for the Care and Use for Laboratory Animals published by the Institute of Laboratory Animal Resources, National Research Council (2010), and were approved by the Merck Research Laboratories Institutional Animal Care and Use Committee. Any and all approved procedures were strictly adhered to. Veterinary care was promptly given to any animal requiring medical attention.

In vivo mouse studies

Housing

Lean C57BL/6 mice at 10-12 weeks of age and C57BL/6 eDIO mice at 16 weeks of age were purchased from Taconic (Germantown, New York). db/db mice at 7 weeks of age were purchased from Jackson Laboratory (Bar Harbor, Maine). Animals were maintained on a 12 hr/12 hr light-dark cycle with free access to food and water with the temperature maintained at 22 ºC. Four lean C57BL/6 mice were housed in a standard cage. eDIO mice were individually caged. C57BL/6 mice were maintained on regular rodent chow diet 7012 (5% dietary fat; 3.75 kcal/g) (Teklad, Madison, WI) for 1-2 weeks before receiving compound treatments. eDIO mice were maintained on 60% kcal% fat diet (Research Diet D12492i). Oral dosing of compound in standard vehicle, or vehicle alone, was performed using 10 mL/kg body weight. The effect of compounds on various metabolic parameters were established by comparison to vehicle treated animals. The data obtained are presented as mean and SEM and the statistical significance vs. vehicle calculated by one-way ANOVA, *$p < 0.05$, **$p<0.01$ and ***$p<0.001$.

Glucose tolerance tests and other glucose homeostasis measurements

Prior to treatment, mice were grouped (N=8 mice per group) to obtain similar average baseline body weight and blood glucose. For glucose tolerance tests, mice were fasted for 6 hr, followed by oral administration of vehicle with or without compound (10 mL/kg for lean mice; 5 mL/kg for obese mice). At 1 hr post treatment, mice were intraperitoneally injected with 20% (w/v) glucose at 2 g/kg body weight for lean mice and 1 g/kg body weight for eDIO and db/db mice; for some experiments, mice were injected with water alone. Blood glucose was measured by glucometer (Accu-Chek, Roche) at -60 (prior to compound administration), 0 (prior to glucose administration) and 20, 40, 60, and 90 min post glucose challenge. Area under the curve (AUC) was calculated from the glucose time curve and net AUC was the AUC of vehicle or MK-8722 treatment after subtracting the AUC of water/no glucose challenge control group. In the chronic db/db mouse study, blood glucose was measured at 24 hr post dosing on days 0, 3, 6, 9, and 12. Plasma insulin was measured using insulin ELISA kit (Mercodia, Sweden). For PK analysis of compound, blood (20 μL) was collected by tail bleeding and mixed with 0.1 M monosodium citrate (60 μL) and assayed as indicated above. The ARRIVE guidelines for reporting in vivo experiments in animal research, published by NC3Rs, were followed.

For xylose absorption, D-(+)-Xylose (Sigma, Cat # X1500) was administered at 1 g/kg together with dextrose. Blood glucose was measured at 0, 20, 40, 60, 90 min. Plasma concentration of D-(+)-Xylose was measured by using D-Xylose Assay Kit (Megazyme, Cat # K-XYLOSE).

pACC quantitation by MSD analysis

Following vehicle or compound treatment, skeletal muscle or liver (~100 mg) was homogenized in 2 mL of tissue lysis buffer (Cell Signaling #9803) containing protease and phosphatase inhibitors (Roche) with a GenoGrinder. The lysates were centrifuged at 14,000 rpm at 4 ºC for 20 min, the supernatants collected and their total protein concentrations quantitated using the BCA protein assay (Thermo Scientific). Phosphorylated Acetyl-CoA Carboxylase (pACC) was quantitated using a Meso Scale Discovery (MSD) assay. For each sample, total protein (20 μg) was added to a MSD streptavidin-coated microplate to immobilize pACC, followed by washing 3 times with TBST buffer (50 mM Tris HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.6). Rabbit anti-mouse pACC (Millipore #07-303) antibody was diluted (1:250) in TBST containing 1% bovine serum albumin, diluted antibody (25 uL) was added to each well, and the plate gently shaken at room temperature for 1 hr. The plate was then washed 3 times with TBST, anti-rabbit sulfo-tag antibody (MSD #R32AB5) was diluted (1:250) in TBST containing 1% bovine serum albumin, diluted antibody (25 uL) was added to each well, and the plate gently shaken at room temperature for 1 hr. The plate was then washed 3 times with TBST, Read buffer T (150 uL; MSD) added to each well, and the plate was read on the MSD instrument. pACC data is presented in absolute values of electrochemiluminescence (ECL) normalized by total lysate protein. Reported values are mean ± S.E.M. and statistical analysis was performed using one way ANOVA vs. vehicle control.