Supporting Information

Non-Acidic Chemotype Possessing N-Acylated Piperidine Moiety as Potent Farnesoid X Receptor (FXR) Antagonists

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1. Analogue 10 and reference compound 1 inhibit the effect of GW4064 in TR FRET binding assay

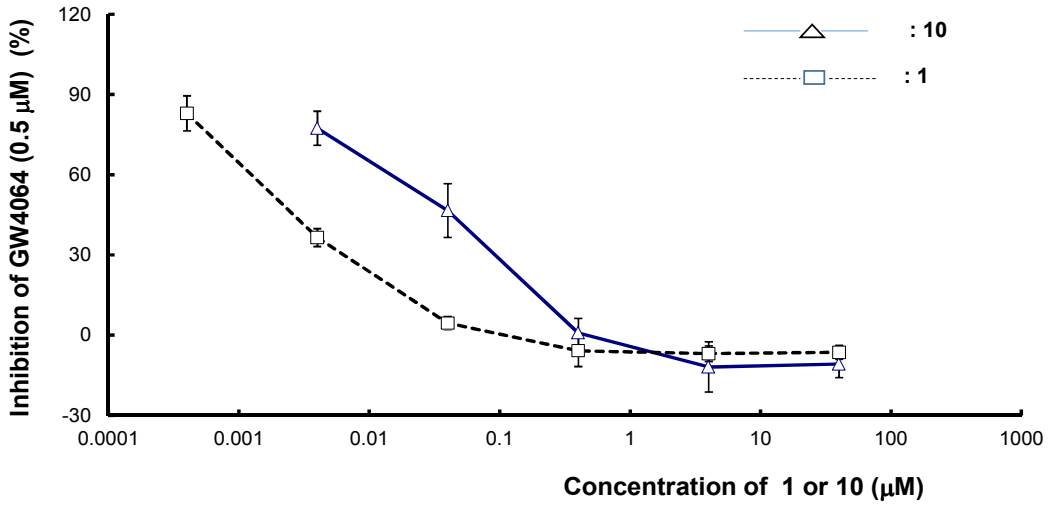


Figure S1. Analogue **10** and reference compound **1** inhibit the effect of GW4064 in TR-FRET binding assay. The method of the assay has been described in the section of experimental procedure.

2. The affinity between 10 and nine nuclear receptors, TGR5

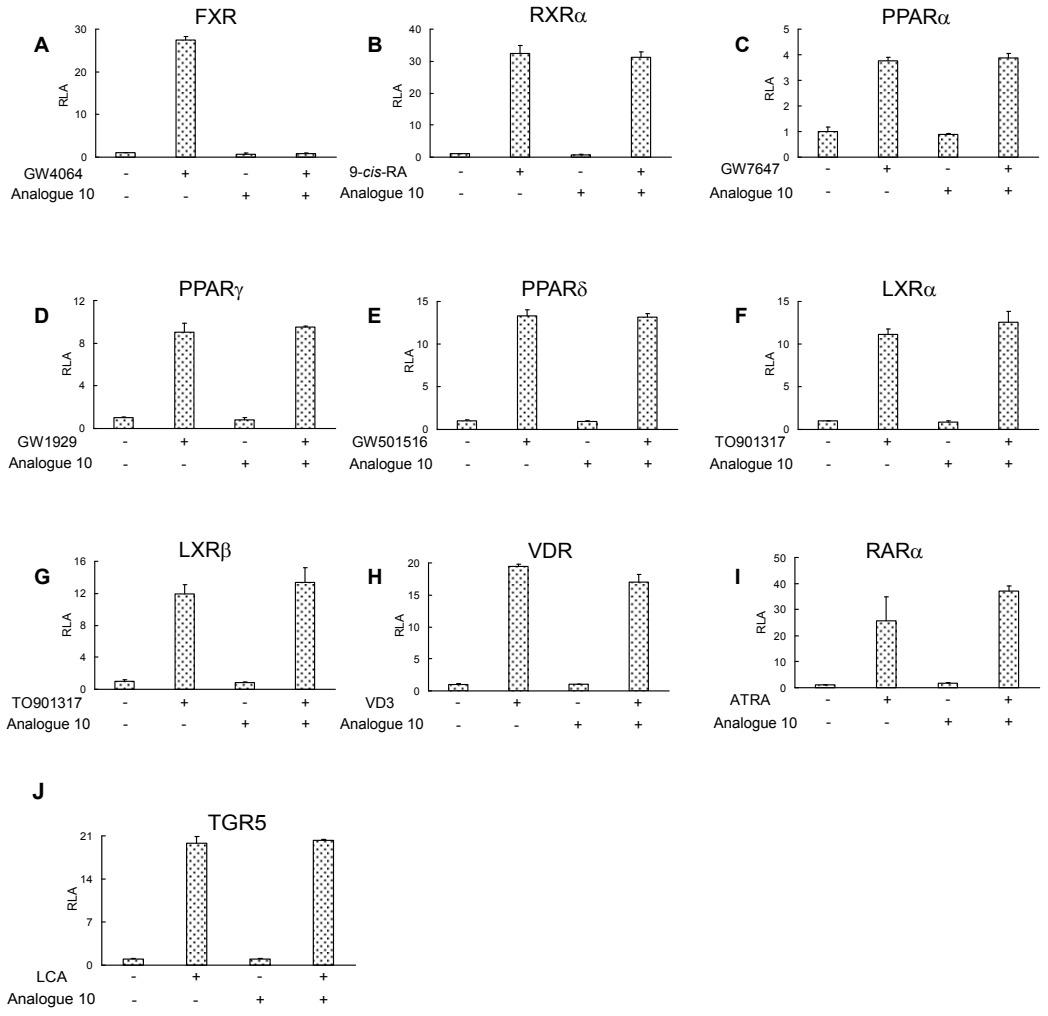


Figure S2. The affinity between **10** and nine nuclear receptors, TGR5. (A) FXR agonist GW4064 (100 nM), (B) RXRα agonist 9-*cis*-retinoic acid (9-*cis*-RA 100 nM), (C) PPARα agonist GW7647 (50 nM), (D) PPARγ agonist GW1929 (50 nM), (E) PPARδ agonist GW501516 (50 nM), (F and G) LXRα and β agonist TO901317 (50 nM), (H) VDR agonist 1,25$(OH)_2$VD3 (VD3, 100 nM), (I) RARα agonist all-trans retinoic acid (ATRA, 100 nM) and (J) TGR5 agonist lithocholic acid (LCA, 200 nM) were as the positive controls. The concentration of analogue **10** was 1 μM in these experiments. RLA: Relative luciferase activity.

3. Effect of 10 on PXR target gene expressions.

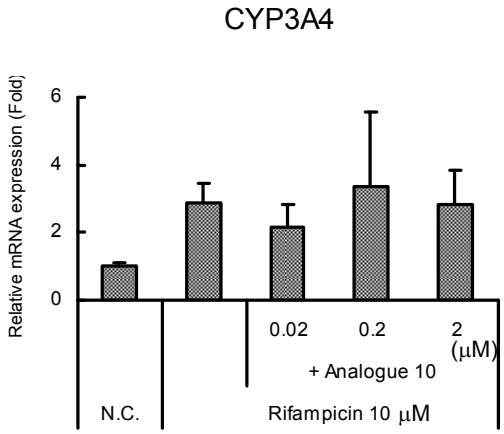


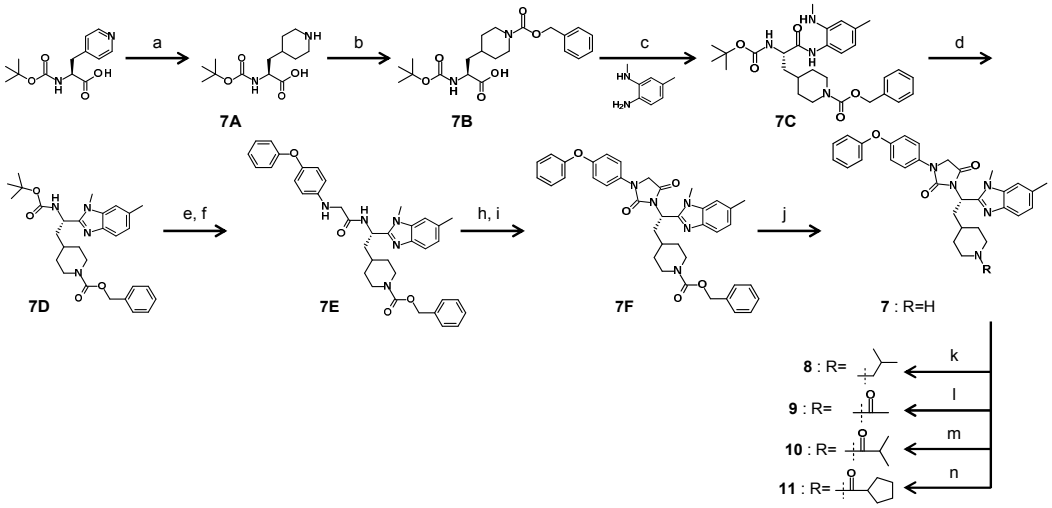
Figure S3. Effect of **10** on PXR target gene expressions. The mRNA expression level of CYP3A4 as PXR target gene was not changed by **10**. HepG2 cells were treated with different concentrations of **10** in the presence of 10 μM Rifampicin for 48 hr. N.C.: Negative control.

Experimentals

General. All chemicals were purchased from Tokyo Chemical Industry Co., Ltd. or Wako Pure Chemical Industries, Ltd. and used without further purification. Amino acid derivative and the coupling reagents were purchased from Watanabe Chemical Industries, Ltd. or Peptide Institute, Inc. ^{1}H-NMR experiments were recorded on a JMTC-600 (JEOL Ltd.) 600 NMR spectrometer with $CDCl_3$, DMSO-d_6, or D_2O as solvents. Chemical shifts are expressed in parts per million (ppm, δ) and referred to the solvent signal. HRMS spectra were recorded on the AccuTOF (JMS-T100LC) equipped with an electrospray ion source (JEOL Ltd.). The analytical HPLC system consisted of a SHINADZU CBM-20A System Controller and a SHIMADZU Pump Unit LC-20AT, a SHIMADZU In-Line Degasser DGU-20A3R, a SHIMADZU SPD-20A Absorbance Detector, a SHIMADZU FCV-11AL Valve Unit and a SHIMADZU FRC-10A Fraction Collector (SHIMADZU Corporation). The absorbance detector was operated at 254 nm. The mobile phase for preparation and analysis was a combination of water (A) and acetonitrile (B), both containing 0.1% TFA, and the flow rate was 8 ml/min and 1 ml/min, respectively. TSK gel ODS columns (21.5 x 300 mm for preparation and 4.6 x 150 mm for analysis, TOSOH Corporation) were used.

4. Experimental procedure and analytical data of analogues 7-11

Scheme S1. Synthesis of Analogues 7-11[a]



[a]Reagents and conditions: (a) PtO_2, H_2, 1 M HCl, MeOH, 24 h, rt; (b) Z-OSu, $NaHCO_3$, CH_3CN/H_2O, 15 h, rt; (c) N2,4-Dimethyl benzene-1,2-diamine, HOAt, WSCI.HCl, DMF, 15 h, -20 °C -> rt; (d) CH_3COOH, 3 h, 80 °C; (e) 4 M HCl/dioxane, 1 h, rt; (f) 2-(4-Phenoxyanilino) acetic acid hydrochloride, HOAt, WSCI.HCl, 15 h, 0 °C -> rt; (h) 4-Nitrophenyl chloroformate, Et_3N, THF, 15 h, rt; (i) 1 M TBAF, THF, 3 h, rt; (j) 10 % Pd/C, H_2, MeOH, 3 h, rt; (k) 1-Bromo-2-methylpropane, K_2CO_3, DMF, 15 h, rt; (l) Acetic anhydride, DMF, Et_3N, 3 h, rt: (m) Isobutyric anhydride, DMF, Et_3N, 3 h, rt: (n) Cyclopentanecarboxylic acid, HOAt, WSCI.HCl, DMF, 15 h, 0 °C -> rt;

(2S)-2-(*tert*-Butoxycarbonylamino)-3-(4-piperidyl)propanoic acid hydrochloride (7A): A suspension of platinum (IV) oxide (72 mg, 0.32 mmol) and (2S)-2-(*tert*-butoxycarbonylamino)-3-(4-pyridyl)propanoic acid (500 mg, 1.88 mmol) in MeOH (20 ml) and 1M HCl (1.88 ml) was stirred under a hydrogen atmosphere at room temperature for 24 h. The mixture was filtered through celite and the filtrate evaporated to afford **7A** in 98 % yield. The product was used in the next reaction without further purification. Rf=0.22 (CH_2Cl_2/MeOH=6/4), ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.78 (br, 1H), 7.09 (m, 1H), 3.91 (m, 1H), 3.23 (m, 5H), 2.81 (m, 2H), 1.83-1.56 (m, 4H), 1.39 (s, 9H), 1.29 (m, 1H). HRMS (ESI/TOF) m/z: $[M + H]^+$ Calcd for $C_{13}H_{25}N_2O_4$ 273.18143 ; Found 273.17973.

(2S)-3-(1-Benzyloxycarbonyl-4-piperidyl)-2-(tert-butoxycarbonylamino)propanoic acid (7B): A mixture of **7A** (1.16 g, 3.77 mmol), N-(benzyloxycarbonyloxy)succinimide (0.98 g, 3.95 mmol), and $NaHCO_3$ (0.95 g, 11.3 mmol) in CH_3CN/H_2O (30 ml/25 ml) was stirred at ambient temperature for 15 h. The organic solvent was removed *in vacuo* and the aqueous layer was washed with ether. The aqueous layer was collected and adjusted pH to 3 with 2 M HCl and the white precipitation was extracted with EtOAc. The combined extracts were washed with H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure to give **7B** in 90 % yield. Rf=0.45 (CH_2Cl_2/MeOH=9/1), ^{1}H NMR (600 MHz, $CDCl_3$): δ 9.80 (br, 1H), 7.38-7.32 (m, 5H), 5.12 (s, 2H), 4.92 (d, 1H), 6.88 (d, J=8.0 Hz, 1H), 4.37 (m, 1H), 4.12 (br, 2H), 2.12 (br, 2H), 1.78-1.59 (m, 4H), 1.45 (s, 9H), 1.15 (m, 2H). HRMS (ESI/TOF) m/z: $[M + Na]^+$ Calcd for $C_{21}H_{30}N_2O_6Na$ 429.20016 ; Found 429.20033.

Benzyl 4-[(2S)-2-(*tert*-butoxycarbonylamino)-3-[4-methyl-2-(methylamino)anilino]-3-oxo-propyl]piperidine-1-carboxylate (7C): To a solution of **7B** (500 mg, 1.23 mmol) in DMF (3 ml), N2,4-dimethylbenzene-1,2-diamine[1] (168 mg, 1.23 mmol) in DMF (3 ml) was added at -20 ^{o}C. Subsequently, HOAt (184 mg, 1.35 mmol) and WSCI.HCl (354 mg, 1.85 mmol) were added to the former solution at -20 ^{o}C and stirred for 15 h at ambient temperature. The reaction mixture was quenched with sat.NH_4Cl and extracted with EtOAc. The combined extracts were washed with H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give **7C** in 71 % yield. Rf=0.50 (*n*-Hexane/EtOAc=1/1), ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.14 (s, 1H), 7.39-7.34 (m, 5H), 7.16 (d, J=6.0 Hz, 1H), 6.88 (d, J=6.6 Hz, 1H), 6.41 (s, 1H), 6.38 (d, J=7.8 Hz, 1H), 5.08 (s, 2H), 4.90 (d, J=4.8 Hz, 1H), 4.10 (m, 1H), 4.02 (m, 2H), 2.83 (m, 1H), 2.75 (m, 1H), 2.71 (d, J=4.2

Hz, 3H), 2.25 (s, 3H), 1.72 (m, 2H), 1.56 (d, J=4.8 Hz, 3H), 1.42 (s, 9H), 1.06 (m, 2H). HRMS (ESI/TOF) m/z: $[M + H]^+$ Calcd for $C_{29}H_{41}N_4O_5$ 525.30769 ; Found 525.30737.

Benzyl 4-[(2S)-2-*(tert*-butoxycarbonylamino)-2-(1,6-dimethylbenzimidazol-2-yl)ethyl] piperidine-1-carboxylate (7D): 7C (450 mg, 0.86 mmol) was dissolved in CH_3COOH (15 ml) at room temperature and stirred for 3 h at 80 ^oC. The reaction mixture was evaporated and quenched by addition of sat.$NaHCO_3$. The mixture was extracted with EtOAc, and organic layer was washed successively with H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give **7D** in 94 % yield. Rf=0.45 (*n*-Hexane/EtOAc=1/1), ^1H NMR (600 MHz, DMSO-d_6): δ 7.45 (t, J=8.4 Hz, 2H), 7.40-7.32 (m, 6H), 7.01 (d, J=7.8 Hz, 1H), 5.07 (s, 2H), 5.00 (m, 1H), 4.03-4.00 (m, 2H), 3.74 (s, 3H), 2.76 (m, 2H), 2.45 (s, 3H), 1.92 (m, 1H), 1.84 (m, 2H), 1.67 (m, 1H), 1.54 (m, 1H), 1.39 (s, 9H), 1.15-1.06 (m, 2H). HRMS (ESI/TOF) m/z: $[M + H]^+$ Calcd for $C_{29}H_{39}N_4O_4$ 507.29713 ; Found 507.29714.

Benzyl 4-[(2S)-2-(1,6-dimethylbenzimidazol-2-yl)-2-[[2-(4-phenoxyanilino)acetyl] amino]ethyl]piperidine-1-carboxylate (7E): To an amorphous powder of **7D** (400 mg, 0.79 mmol), 4M HCl/dioxane (3.95 ml, 15.8 mmol) was added at room temperature and the mixture was stirred for 1 h at ambient temperature. Ether was added to the reaction mixture to yield the precipitate. The product was filtered and dried over for 3h *in vacuo*. The product in DMF (3 ml) was added to a solution of 2-[(4-phenoxyphenyl)amino]acetic acid hydrochloride[2] (221 mg, 0.79 mmol) and triethylamine (332 μl, 2.37 mmol) in DMF (2 ml) at 0 ^oC. Subsequently, HOAt (118 mg, 0.87 mmol) and WSCI.HCl (228 mg, 1.19 mmol) were added to the former solution at 0 ^oC and stirred for 15h at ambient temperature. The reaction mixture was quenched with sat.NH_4Cl and extracted with EtOAc. The combined extracts were washed with H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give **7E** in 74 % yield. Rf=0.45 (*n*-Hexane/EtOAc=1/4), ^1H NMR (600 MHz, DMSO-d_6): δ 8.43 (d, J=9.0 Hz, 1H), 7.44 (d, J=7.2 Hz, 1H), 7.45-7.24 (m, 8H), 6.97 (m, 2H), 6.79 (d, J=8.4 Hz, 4H), 6.55 (d, J=9.0 Hz, 2H), 5.94 (m, 1H), 5.33 (m, 1H), 5.03 (s, 2H), 3.91 (brs, 2H), 3.69-3.66 (m, 2H), 3.66 (s, 3H), 2.63 (m, 2H), 2.40 (s, 3H), 1.91 (m, 1H), 1.79 (m, 2H), 1.52 (m, 1H), 1.42 (m, 1H), 1.07 (m, 1H), 0.98 (m, 1H). HRMS (ESI/TOF) m/z: $[M + Na]^+$ Calcd for $C_{38}H_{41}N_5O_4Na$ 654.30562 ; Found 654.30510.

Benzyl 4-[(2S)-2-(1,6-dimethylbenzimidazol-2-yl)-2-[2,5-dioxo-3-(4-phenoxyphenyl) imidazolidin-1-yl]ethyl]piperidine-1-carboxylate (7F): At room temperature, triethylamine

(406 µl, 2.90 mmol) was added to **7E** (365 mg, 0.58 mmol) in dist.THF (5 ml) under a N_2 atmosphere. After stirring for 5 min, to the mixture, 4-nitrophenyl choloformate (234 mg, 1.16 mmol) was added at ambient temperature and stirred for 15 h under the same conditions. The reaction mixture was quenched with sat.$NaHCO_3$ and extracted with EtOAc. The combined extracts were washed H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure. To the residue in THF (3 ml), TBAF (1M THF solution) (1.74 ml, 1.74 mmol) was added at ambient temperature and stirred for 3 h under the same conditions. The reaction mixture was quenched with sat.NH_4Cl and extracted with EtOAc. The combined extracts were washed with sat.$NaHCO_3$, H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give **7F** in 71 % yield. Rf=0.70 (*n*-Hexane/EtOAc=1/2), ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.65 (d, J=8.4 Hz, 2H), 7.52 (d, J=7.8 Hz, 1H), 7.40-7.33 (m, 8H), 7.13 (t, J=8.4 Hz, 1H), 7.09 (d, J=7.8 Hz, 2H), 7.03 (d, J=8.4 Hz, 1H), 6.98 (d, J=7.8 Hz, 2H), 5.58 (brs, 1H), 5.08 (s, 2H), 4.60 (s, 2H), 4.02 (brs, 2H), 3.68 (s, 3H), 2.84 (m, 1H), 2.77 (m, 1H), 2.52 (m, 1H), 2.45 (m, 3H), 2.33 (m, 1H), 1.93 (m, 1H), 1.69 (m, 1H), 1.63 (m, 1H), 1.19 (m, 1H), 1.14 (m, 1H). HRMS (ESI/TOF) m/z: $[M + H]^+$ Calcd for $C_{39}H_{40}N_5O_5$ 658.30294 ; Found 658.30318.

3-[(1S)-1-(1,6-Dimethylbenzimidazol-2-yl)-2-(4-piperidyl)ethyl]-1-(4-phenoxyphenyl)imidazolidine-2,4-dione (7): To **7F** (200 mg, 0.30 mmol) and 10 % Pd/C (65.5 mg), was added dist.MeOH (10 ml) and hydrogenated under a hydrogen atmosphere for 3 h at ambient temperature. The solution was filtered and concentrated to provide a white solid. The products were purified by HPLC (column: TSK gel ODS-120T, Φ 21.5 x 300 mm, eluent CH_3CN containing 0.1% TFA/H_2O containing 0.1% TFA) to give **7** in 79% yield, Rf=0.20 (CH_2Cl_2/MeOH=8/2), ^{1}H NMR (600 MHz, D_2O): δ 7.49 (d, J=9.0 Hz, 1H), 7.35 (s, 1H), 7.28 (d, J=9.0 Hz, 1H), 7.17 (d, J=9.0 Hz, 2H), 7.10 (t, J=8.4 Hz, 2H), 6.91 (t, J=7.2 Hz, 1H), 6.74 (d, J=9.0 Hz, 2H), 6.68 (d, J=8.4 Hz, 2H), 5.77 (dd, J=10.2, 4.8 Hz, 1H), 4.35 (q, J=18.0 Hz, 2H), 3.78 (s, 3H), 3.28 (brt, J=13.8 Hz, 2H), 2.78 (m, 2H), 2.65 (m, 1H), 2.31 (s, 3H), 2.13 (m, 1H), 2.03 (brd, J=13.8 Hz, 1H), 1.82 (brd, J=14.4 Hz, 1H), 1.60 (m, 1H), 1.44 (m, 1H), 1.35 (m, 1H). ^{13}C NMR (600 MHz, D_2O): δ 171.0, 163.6, 157.0, 154.9, 154.1, 146.59, 146.58, 138.7, 133.1, 132.1, 130.6, 129.6, 124.6, 122.6, 119.9, 119.3, 114.2, 112.6, 51.3, 44.2, 44.1, 43.1, 34.4, 31.9, 30.1, 28.9, 27.8, 21.4. HRMS (ESI/TOF) m/z: $[M + Na]^+$ Calcd for $C_{31}H_{33}N_5O_3Na$ 546.24811 ; Found 546.24669.

3-[(1S)-1-(1,6-Dimethylbenzimidazol-2-yl)-2-(1-isobutyl-4-piperidyl)ethyl]-1-(4-phenoxyphenyl)imidazolidine-2,4-dione (8): To a solution of **7** (55.0 mg, 0.11 mmol) in DMF (3 ml), K_2CO_3 (30.4 mg, 0.22 mmol) and 1-bromo-2-methylpropane (16.4 mg, 0.12 mmol) were added

at ambient temperature and stirred for 15 h. The reaction mixture was quenched with sat. NH_4Cl and extracted with EtOAc. The combined extracts were washed with H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give **8** in 30% yield. Rf = 0.35 (EtOAc/MeOH = 9/1), ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.82 (d, J=7.8 Hz, 1H), 7.45 (d, J=9.0 Hz, 2H), 7.33 (m, 3H), 7.23 (brs, 1H), 7.11 (t, J=7.8 Hz, 1H), 7.00 (d, J=9.0 Hz, 2H), 6.97 (d, J=8.4 Hz, 2H), 5.75 (t, J=6.6 Hz, 1H), 4.42 (brs, 2H), 3.95 (s, 3H), 3.59 (d, J=11.4 Hz, 1H), 3.53 (d, J=10.8 Hz, 1H), 2.90-2.57 (m, 7H), 2.53 (s, 3H), 2.06 (quint., J=6.6 Hz, 1H), 2.13 (d, J=13.2 Hz, 1H), 1.93 (m, 3H), 0.99 (t, J=7.2 Hz, 6H). ^{13}C NMR (600 MHz, $CDCl_3$): δ 167.9, 161.6, 157.0, 154.5, 152.7, 147.2, 147.1, 137.2, 133.3, 131.9, 129.9, 128.2, 123.5, 120.8, 119.73, 119.66, 118.8, 116.5, 110.6, 64.7, 53.1, 50.3, 43.9, 34.5, 31.4, 30.2, 28.2, 27.8, 23.9, 21.9, 20.32, 20.25., HRMS (ESI/TOF) m/z: $[M + Na]^+$ Calcd for $C_{35}H_{41}N_5O_3Na$ 602.31071 ; Found 602.30996.

3-[(1S)-2-(1-Acetyl-4-piperidyl)-1-(1,6-dimethylbenzimidazol-2-yl)ethyl]-1-(4-phenoxyphenyl)imidazolidine-2,4-dione (9): To a solution of **7** (159 mg, 0.24 mmol) in DMF (3 ml), triethylamine (67.3 µl, 0.48 mmol) and acetic anhydride (24.5 µl, 0.26 mmol) were added at ambient temperature and stirred for 3 h. The reaction mixture was quenched with sat. NH_4Cl and extracted with EtOAc. The combined extracts were washed with H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give **9** in 87% yield. Rf = 0.80 (EtOAc/MeOH = 9/1), ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.71 (dd, J=8.4, 4.8 Hz, 1H), 7.51 (dd, J=8.4, 3.6 Hz, 2H), 7.34 (t, J=7.8 Hz, 2H), 7.11 (t, J=7.8 Hz, 2H), 7.10 (t, J=8.4 Hz, 1H), 7.04 (d, J=9.0 Hz, 2H), 6.98 (d, J=8.4 Hz, 2H), 5.61 (m, 1H), 4.61 (t, J=14.4 Hz, 1H), 4.33 (s, 2H), 3.81 (m, 1H), 3.76 (s, 3H), 3.01 (td, J=12.6, 6.6 Hz, 1H), 2.87 (td, J=10.2, 4.8 Hz, 0.5H), 2.68 (m, 0.5H), 2.60 (m, 0.5H), 2.52 (m, 1H), 2.49 (s, 3H), 2.39 (m, 0.5H), 2.08 (s, 3H), 2.01 (m, 1H), 1.80 (m, 1H), 1.64 (m, 1H), 1.32-1.20 (m, 2H). ^{13}C NMR (600 MHz, $CDCl_3$): δ 168.8, 167.6, 167.5, 157.1, 154.1, 153.5, 149.6, 149.5, 140.2, 136.1, 133.3, 132.5, 129.8, 124.0, 123.5, 120.2, 119.97, 119.94, 119.8, 118.6, 109.2, 49.8, 46.4, 45.5, 41.5, 36.2, 33.0, 32.8, 32.2, 31.8, 31.0, 30.0, 21.9, 21.5. HRMS (ESI/TOF) m/z: $[M + H]^+$ Calcd for $C_{33}H_{36}N_5O_4$ 566.27673 ; Found 566.27373.

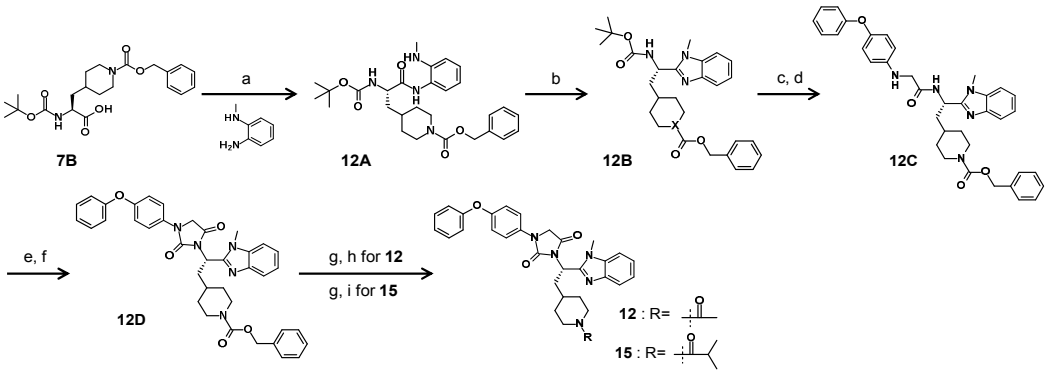
3-[(1S)-1-(1,6-Dimethylbenzimidazol-2-yl)-2-[1-(2-methylpropanoyl)-4-piperidyl]ethyl]-1-(4-phenoxyphenyl)imidazolidine-2,4-dione (10): To a solution of **7** (60.0 mg, 0.09 mmol) in DMF (3 ml), triethylamine (25.5 µl, 0.18 mmol) and isobutyric anhydride (18.2 µl, 0.11 mmol) were added at ambient temperature and stirred for 3 h. The reaction mixture was quenched with sat. NH_4Cl and extracted with EtOAc. The combined extracts were washed with H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica

gel column chromatography to give **10** in 77% yield. Rf = 0.50 (*n*-hexane/EtOAc = 1/2), ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.75 (d, J=7.2 Hz, 1H), 7.51 (dd, J=7.8, 3.0 Hz, 2H), 7.33 (t, J=7.8 Hz, 2H), 7.13 (brs, 2H), 7.11 (t, J=7.8 Hz, 1H), 7.04 (d, J=9.0 Hz, 2H), 6.98 (d, J=7.8 Hz, 2H), 5.61 (m, 1H), 4.65 (t, J=15.0 Hz, 1H), 4.33 (s, 2H), 3.94 (brd, J=13.2 Hz, 1H), 3.78 (s, 3H), 3.00 (m, 1H), 2.90 (m, 0.5H), 2.79 (quint., J=6.0 Hz, 1H), 2.67-2.62 (m, 1H), 2.53 (m, 1H), 2.50 (s, 3H), 2.37 (m, 0.5H), 2.04 (brt, J=15.0 Hz, 1H), 1.81 (m, 1H), 1.63 (m, 1H), 1.32-1.18 (m, 2H), 1.10 (m, 6H)., ^{13}C NMR (600 MHz, $CDCl_3$): δ 175.3, 167.7, 167.6, 157.1, 154.2, 153.4, 149.4, 149.2, 140.2, 135.8, 133.7, 132.5, 129.8, 124.4, 123.5, 120.3, 119.8, 119.7, 118.6, 109.3, 49.8, 45.4, 41.8, 36.1, 33.2, 33.0, 32.5, 31.8, 31.1, 30.1, 21.9, 19.6, 19.3. HRMS (ESI/TOF) m/z: $[M + H]^+$ Calcd for $C_{35}H_{40}N_5O_4$ 594.30803 ; Found 594.30726.

3-[(1S)-2-[1-(Cyclopentanecarbonyl)-4-piperidyl]-1-(1,6-dimethylbenzimidazol-2-yl)ethyl]-1-(4-phenoxyphenyl)imidazolidine-2,4-dione (11): Compound **7** (55.0 mg, 0.11 mmol) in DMF (1 ml) was added to a solution of cyclopentanecarboxylic acid (13.7 mg, 0.12 mmol) in DMF (2 ml) at 0 ^{o}C. Subsequently, HOAt (17.7 mg, 0.13 mmol) and WSCI.HCl (32.6 mg, 0.17 mmol) were added to the former solution at 0 ^{o}C and stirred for 15h at ambient temperature. The reaction mixture was quenched with sat.NH_4Cl and extracted with EtOAc. The combined extracts were washed with H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give **11** in 21 % yield. Rf=0.80 (EtOAc), ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.73 (d, J=4.8 Hz, 1H), 7.51 (dd, J=8.4, 4.2 Hz, 2H), 7.33 (t, J=7.2 Hz, 2H), 7.11 (brs, 2H), 7.10 (brs, 1H), 7.04 (d, J=9.0 Hz, 2H), 6.98 (d, J=8.4 Hz, 2H), 5.61 (m, 1H), 4.64 (t, J=16.8 Hz, 1H), 4.33 (d, J=7.8 Hz, 2H), 3.97 (d, J=12.6 Hz, 1H), 3.76 (s, 3H), 2.98 (m, 1H), 2.90 (m, 0.5H), 2.88 (quint., J=7.2 Hz, 1H), 2.68 (m, 0.5H), 2.60 (m, 0.5H), 2.51 (m, 1H), 2.49 (s, 3H), 2.37 (m, 0.5H), 2.01 (m, 1H), 1.81-1.55 (m, 10H), 1.30-1.17 (m, 2H). ^{13}C NMR (600 MHz, $CDCl_3$): δ 174.3, 167.7, 167.5, 157.1, 154.1, 153.4, 149.6, 149.4, 139.8, 136.0, 133.4, 132.5, 129.83, 129.81, 124.2, 123.5, 120.21, 120.19, 119.8, 118.6, 109.2, 49.8, 45.5, 41.9, 41.1, 36.1, 33.2, 33.1, 33.0, 32.4, 31.9, 31.1, 30.2, 30.0. HRMS (ESI/TOF) m/z: $[M + Na]^+$ Calcd for $C_{37}H_{41}N_5O_4Na$ 642.30562 ; Found 642.30572.

5. Experimental procedure and analytical data of analogues 12 and 15

Scheme S2. Synthesis of Analogues 12 and 15[a]



[a]Reagents and conditions: (a) N2-Methylbenzene-1,2-diamine, HOAt, WSCI.HCl, DMF, 15 h, -20 °C -> rt; (b) CH_3COOH, 3 h, 80 °C; (c) 4 M HCl/dioxane, 1 h, rt; (d) 2-(4-Phenoxyanilino) acetic acid hydrochloride, HOAt, WSCI.HCl, 15 h, 0 °C -> rt; (e) 4-Nitrophenyl chloroformate, Et_3N, THF, 15 h, rt; (f) 1 M TBAF, THF, 3 h, rt; (g) 10 % Pd/C, H_2, MeOH, 5 h, rt; (h) Acetic anhydride, CH_2Cl_2, Et_3N, 3 h, rt: (i) Isobutyric anhydride, CH_2Cl_2, Et_3N, 3 h, rt:

Benzyl 4-[(2S)-2-(tert-butoxycarbonylamino)-3-[2-(methylamino)anilino]-3-oxo-propyl] piperidine-1-carboxylate (12A): To a solution of **7B** (790 mg, 1.96 mmol) in DMF (10 ml), N2-methylbenzene-1,2-diamine[3] (240 mg, 1.96 mmol) was added at -20 °C. Subsequently, HOAt (310 mg, 2.35 mmol) and WSCI.HCl (450 mg, 2.35 mmol) were added to the former solution at -20 °C and stirred for 15 h at ambient temperature. The reaction mixture was quenched with sat.NH_4Cl and extracted with EtOAc. The combined extracts were washed with H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give **12A** in 71 % yield. Rf=0.60 (*n*-Hexane/EtOAc=1/2), ^1H NMR (600 MHz, $CDCl_3$): δ 7.64 (br, 1H), 7.39-7.30 (m, 6H), 7.26-7.24 (m, 1H), 7.18-7.16 (m, 1H), 6.74-6.71 (m, 2H), 5.13 (s, 2H), 5.12 (br, 1H), 4.96 (br, 1H), 4.22-4.17 (m, 3H), 2.85-2.79 (m, 2H), 2.83 (s, 3H), 1.90-1.56 (m, 4H), 1.47 (s, 9H), 1.44-1.43 (m, 2H). HRMS (ESI/TOF) m/z: $[M + H]^+$ Calcd for $C_{28}H_{39}N_4O_5$ 511.29204 ; Found 511.28999.

Benzyl 4-[(2S)-2-(tert-butoxycarbonylamino)-2-(1-methylbenzimidazol-2-yl)ethyl] piperidine-1-carboxylate (12B): 12A (840 mg, 1.64 mmol) was dissolved in CH_3COOH (6 ml) at room temperature and stirred for 3 h at 80 °C. The reaction mixture was evaporated and quenched by addition of sat.$NaHCO_3$. The mixture was extracted with EtOAc, and organic layer was washed successively with H_2O, and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give **12B** in 93 % yield. Rf=0.55 (*n*-Hexane/EtOAc=1/2), ^1H NMR (600 MHz, $CDCl_3$): δ 7.75 (d, J=7.9 Hz,

1H), 7.38-7.30 (m, 8H), 5.20-5.14 (m, 4H), 4.16 (m, 2H), 3.84 (s, 3H), 2.77 (br, 2H), 2.00-1.94 (m, 3H), 1.67-1.61 (m, 2H), 1.45 (s, 9H), 1.29-1.19 (m, 2H). HRMS (ESI/TOF) m/z: $[M + Na]^+$ Calcd for $C_{28}H_{36}N_4O_4Na$ 515.26342 ; Found 515.26206.

Benzyl 4-[(2S)-2-(1-methylbenzimidazol-2-yl)-2-[[2-(4-phenoxyanilino)acetyl]amino] ethyl]piperidine-1-carboxylate (12C): To an amorphous powder of **12B** (350 mg, 0.71 mmol), 4M HCl/dioxane (1.7 ml, 7.1 mmol) was added at room temperature and the mixture was stirred for 1 h at ambient temperature. Ether was added to the reaction mixture to yield the precipitate. The product was filtrated and dried over for 3h *in vacuo*. The product in DMF (8 ml) was added to a solution of 2-[(4-phenoxyphenyl)amino]acetic acid hydrochloride[2] (200 mg, 0.71 mmol) and triethylamine (110 μl, 0.78 mmol) in DMF (8 ml) at 0 °C. Subsequently, HOAt (110 mg, 0.85 mmol) and WSCI.HCl (160 mg, 0.85 mmol) were added to the former solution at 0 °C and stirred for 15 h at ambient temperature. The reaction mixture was quenched with sat.NH_4Cl and extracted with EtOAc. The combined extracts were washed with H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give **12C** in 25 % yield. Rf=0.75 (*n*-Hexane/EtOAc=1/4), ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.72 (d, J=7.9 Hz, 1H), 7.38-7.32 (m, 11H), 7.04 (t, J=7.6 Hz, 1H), 6.91 (d, J=8.7 Hz, 4H), 6.58 (d, J=8.6 Hz, 2H), 5.61-5.58 (m, 1H), 5.13 (s, 2H), 4.21 (m, 3H), 3.87 (s, 3H), 3.84-3.83 (br, 2H), 2.72 (m, 2H), 2.02-1.90 (m, 3H), 1.49 (m, 2H), 1.18 (br, 2H). HRMS (ESI/TOF) m/z: $[M + Na]^+$ Calcd for $C_{37}H_{39}N_5O_4Na$ 640.28997 ; Found 640.29003.

Benzyl 4-[(2S)-2-[2,5-dioxo-3-(4-phenoxyphenyl)imidazolidin-1-yl]-2-(1-methylbenz-imidazol-2-yl)ethyl]piperidine-1-carboxylate (12D): At room temperature, triethylamine (100 μl, 0.71 mmol) was added to **12C** (110 mg, 0.18 mmol) in dist.THF (5 ml) under a N_2 atmosphere. After stirring for 5 min, to the mixture, 4-nitrophenyl choloformate (72 mg, 0.36 mmol) was added at ambient temperature and stirred for 15 h under the same conditions. The reaction mixture was quenched with sat.$NaHCO_3$ and extracted with EtOAc. The combined extracts were washed H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure. To the residue in THF (3 ml), TBAF (1M THF solution) (0.71 ml, 0.71 mmol) was added at ambient temperature and stirred for 15 h under the same conditions. The reaction mixture was quenched with sat.NH_4Cl and extracted with EtOAc. The combined extracts were washed with sat.$NaHCO_3$, H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give **12D** in 70 % yield. Rf=0.62 (*n*-Hexane/EtOAc=1/4), ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.84 (d, J=7.4 Hz, 1H), 7.51-7.50 (m, 2H), 7.36-7.28 (m, 10H), 7.10 (t, J=7.4 Hz, 1H), 7.04 (d, J=9.1 Hz, 2H), 6.97 (d,

J=7.7 Hz, 2H), 5.63-5.60 (m, 1H), 5.11 (s, 2H), 4.32 (s, 2H), 4.17 (m, 2H), 3.80 (s, 3H), 2.76 (m, 3H), 2.42 (m, 2H), 1.97 (br, 1H), 1.74 (br, 1H), 1.31-1.22 (m, 2H). HRMS (ESI/TOF) m/z: $[M + H]^+$ Calcd for $C_{38}H_{38}N_5O_5$ 644.28729 ; Found 644.28529.

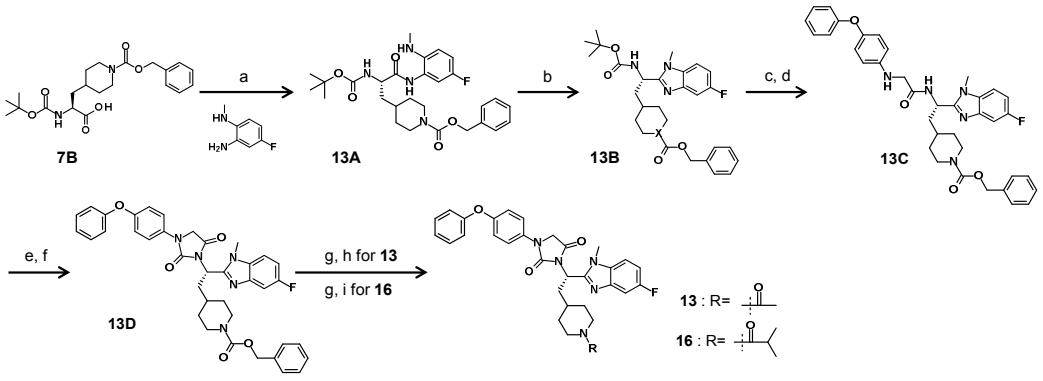
3-[(1S)-2-(1-Acetyl-4-piperidyl)-1-(1-methylbenzimidazol-2-yl)ethyl]-1-(4-phenoxy-phenyl)imidazolidine-2,4-dione (12): To **12D** (45 mg, 0.07 mmol) and 10 % Pd/C (50 mg), was added dist.MeOH (3 ml) and hydrogenated under a hydrogen atmosphere for 3 h at ambient temperature. The solution was filtered and concentrated to provide a white solid. To a solution of the obtained solid in CH_2Cl_2 (3 ml), triethylamine (59 µl, 0.42 mmol) and acetic anhydride (33 µl, 0.35 mmol) were added at ambient temperature and stirred for 3 h. The reaction mixture was quenched with sat. NH_4Cl and extracted with EtOAc. The combined extracts were washed with H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give **12** in 43% yield. Rf=0.80 (EtOAc/MeOH=9/1), ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.84 (t, J=7.2 Hz, 1H), 7.51 (dd, J=8.4, 3.6 Hz, 2H), 7.35-7.27 (m, 5H), 7.11 (t, J=6.6 Hz, 1H), 7.04 (d, 9.0 Hz, 2H), 6.98 (d, J=8.4 Hz, 2H), 5.62 (td, J=10.2, 5.4 Hz, 1H), 4.62 (t, J=14.4 Hz, 1H), 4.33 (d, J=6.6 Hz, 2H), 3.82 (m, 1H), 3.80 (s, 3H), 3.02 (td, J=13.2, 5.4 Hz, 1H), 2.89 (m, 0.5H), 2.69 (m, 0.5H), 2.60 (m, 0.5H), 2.53 (q, J=12.6 Hz, 1H), 2.40 (m, 0.5H), 2.08 (s, 3H), 2.02 (m, 1H), 1.81 (m, 1H), 1.62 (m, 1H), 1.33-1.19 (m, 2H)., ^{13}C NMR (600 MHz, $CDCl_3$): δ 168.8, 167.6, 167.5, 157.1, 154.1, 153.4, 150.1, 150.0, 142.1, 135.9, 132.5, 129.8, 123.5. 123.2, 122.4, 120.4, 120.1, 119.8, 118.6, 109.3, 49.7, 46.4, 45.5, 41.5, 36.1, 32.9, 32.8, 32.1, 31.7, 31.0, 21.5., HRMS (ESI/TOF) m/z: $[M + Na]^+$ Calcd for $C_{32}H_{33}N_5O_4Na$ 574.24302 ; Found 574.24371.

3-[(1S)-1-(1-Methylbenzimidazol-2-yl)-2-[1-(2-methylpropanoyl)-4-piperidyl]ethyl]-1-(4-phenoxyphenyl)imidazolidine-2,4-dione (15): To **12D** (45 mg, 0.07 mmol) and 10 % Pd/C (50 mg), was added dist.MeOH (3 ml) and hydrogenated under a hydrogen atmosphere for 3 h at ambient temperature. The solution was filtered and concentrated to provide a white solid. To a solution of the obtained solid in CH_2Cl_2 (3 ml), triethylamine (59 µl, 0.42 mmol) and isobutylic anhydride (58 µl, 0.35 mmol) were added at ambient temperature and stirred for 3 h. The reaction mixture was quenched with sat. NH_4Cl and extracted with EtOAc. The combined extracts were washed with H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give **15** in 26% yield. Rf=0.42 (EtOAc), ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.85 (t, J=5.4 Hz, 1H), 7.51 (brd, J=6.0 Hz, 2H), 7.35-7.27 (m, 5H), 7.11 (t, J=6.6 Hz, 1H), 7.04 (d, J=9.0 Hz, 2H), 6.98 (d, J=9.0 Hz, 2H), 5.63 (q, J=6.0 Hz, 1H), 4.66 (brt, J=15.0 Hz, 1H), 4.33 (d, J=6.0 Hz, 2H), 3.94 (brd, J=12.0 Hz,

1H), 3.80 (s, 3H), 3.00 (m, 1H), 2.89 (m, 0.5H), 2.79 (quint., J=6.0 Hz, 1H), 2.71 (m, 0.5H), 2.61 (m, 0.5H), 2.52 (q, J=12.0 Hz, 1H), 2.39 (m, 0.5H), 2.03 (brt, J=9.6 Hz, 1H), 1.81 (m, 1H), 1.66 (m, 1H), 1.30-1.20 (m, 2H), 1.11 (dd, J=13.2, 6.6 Hz, 6H). ^{13}C NMR (600 MHz, $CDCl_3$): δ 175.2, 167.7, 167.5, 157.1, 154.1, 153.4, 150.2, 150.0, 142.1, 135.9, 132.5, 129.8, 123.4, 123.2, 122.4, 120.4, 120.2, 119.8, 118.6, 109.3, 49.7, 45.5, 41.8, 36.2, 33.1, 33.0, 32.5, 31.9, 31.1, 30.1, 19.6, 19.3., HRMS (ESI/TOF) m/z: $[M + H]^+$ Calcd for $C_{34}H_{38}N_5O_4$ 580.29238 ; Found 580.29457.

6. Experimental procedure and analytical data of analogues 13 and 16

Scheme S3. Synthesis of Analogues 13 and 16[a]



[a]Reagents and conditions: (a) 4-Fluoro-N1-methyl-benzene-1,2-diamine, HOAt, WSCI.HCl, DMF, 15 h, -20 °C -> rt; (b) CH_3COOH, 2 h, 80 °C; (c) 4 M HCl/dioxane, 1 h, rt; (d) 2-(4-Phenoxyanilino) acetic acid hydrochloride, HOAt, WSCI.HCl, 15 h, 0 °C -> rt; (e) 4-Nitrophenyl chloroformate, Et_3N, THF, 15 h, rt; (f) 1 M TBAF, THF, 3 h, rt; (g) 10 % Pd/C, H_2, MeOH, 3 h, rt; (h) Acetic anhydride, CH_2Cl_2, Et_3N, 3 h, rt: (i) Isobutyric anhydride, CH_2Cl_2, Et_3N, 3 h, rt:

Benzyl 4-[(2S)-2-(tert-butoxycarbonylamino)-3-[5-fluoro-2-(methylamino)anilino]-3-oxo-propyl]piperidine-1-carboxylate (13A): To a solution of **7B** (1.0 g, 2.46 mmol) in CH_3CN (30 ml), 4-fluoro-N2-methyl-benzene-1,2-diamine[4] (0.34 g, 2.46 mmol) was added at -20 °C. Subsequently, HOAt (0.35 g, 2.7 mmol) and WSCI.HCl (0.56 g, 2.95 mmol) were added to the former solution at -20 °C and stirred for 15 h at ambient temperature. The reaction mixture was quenched with sat.NH_4Cl and extracted with EtOAc. The combined extracts were washed with H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give **13A** in 49 % yield. Rf=0.42 (*n*-Hexane/EtOAc=1/1), ^1H NMR (600 MHz, $CDCl_3$): δ 7.91 (s, 1H), 7.37-7.36 (m, 5H), 7.32-7.31 (m, 2H), 8.87-6.84 (m, 1H), 6.68 (br, 1H), 5.13 (s, 2H), 4.91 (br, 1H), 4.21-4.20 (m, 3H), 3.69 (br, 1H), 2.80 (s, 3H), 2.78 (m, 2H), 1.91-1.72 (m, 4H), 1.47 (s, 9H), 1.25 (m, 2H). HRMS (ESI/TOF) m/z: $[M + H]^+$ Calcd for $C_{28}H_{38}F_1N_4O_5$ 529.28262 ; Found 529.28265.

Benzyl 4-[(2S)-2-(tert-butoxycarbonylamino)-2-(5-fluoro-1-methyl-benzimidazol-2-yl) ethyl]piperidine-1-carboxylate (13B): 13A (0.64 g, 1.2 mmol) was dissolved in CH_3COOH (10 ml) at room temperature and stirred for 2 h at 80 °C. The reaction mixture was evaporated and quenched by addition of sat.$NaHCO_3$. The mixture was extracted with EtOAc, and organic layer was washed successively with H_2O, and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give **13B** in 98 % yield. Rf=0.22 (*n*-Hexane/Ether=1/3), ^1H NMR (600 MHz, $CDCl_3$): δ 7.43-7.31 (m, 6H), 7.26-7.25 (m, 1H), 7.07-7.03 (m, 1H), 5.15-5.11 (m, 4H), 4.14 (br, 2H), 3.81 (s, 3H), 2.75 (m,

2H), 1.95-1.88 (m, 3H), 1.60 (m, 2H), 1.43 (s, 9H), 1.24 (m, 2H). HRMS (ESI/TOF) m/z: $[M + H]^+$ Calcd for $C_{28}H_{36}F_1N_4O_4$ 511.27206 ; Found 511.27401.

Benzyl 4-[(2S)-2-(5-fluoro-1-methyl-benzimidazol-2-yl)-2-[[2-(4-phenoxyanilino)acetyl] amino]ethyl]piperidine-1-carboxylate (13C): To an amorphous powder of **13B** (0.59 g, 1.16 mmol), 4M HCl/dioxane (2.9 ml, 11.6 mmol) was added at room temperature and the mixture was stirred for 1 h at ambient temperature. Ether was added to the reaction mixture to yield the precipitate. The product was filtrated and dried over for 3 h *in vacuo*. The product in DMF (15 ml) was added to a solution of 2-[(4-phenoxyphenyl)amino]acetic acid hydrochloride[2] (0.32 g, 1.16 mmol) and triethylamine (0.36 ml, 2.55 mmol) in DMF (15 ml) at 0 ^oC. Subsequently, HOAt (0.18 g, 1.4 mmol) and WSCI.HCl (0.27 g, 1.4 mmol) were added to the former solution at 0 ^oC and stirred for 15 h at ambient temperature. The reaction mixture was quenched with sat.NH_4Cl and extracted with EtOAc. The combined extracts were washed with H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give **13C** in 78 % yield. Rf=0.38 (*n*-Hexane/EtOAc=1/2), ^1H NMR (600 MHz, $CDCl_3$): δ 7.38-7.31 (m, 8H), 7.26-7.25 (m, 2H), 7.09-7.02 (m, 2H), 6.90 (d, J=8.8 Hz, 4H), 6.56 (d, J=8.8 Hz, 2H), 5.55-5.53 (m, 1H), 5.12 (s, 2H), 4.12 (m, 3H), 3.85 (s, 3H), 3.82-3.79 (m, 2H), 2.71 (br, 2H), 1.99-1.86 (m, 3H), 1.46 (m, 2H), 1.16 (m, 2H). HRMS (ESI/TOF) m/z: $[M + H]^+$ Calcd for $C_{37}H_{39}F_1N_5O_4$ 636.29861 ; Found 636.29856.

Benzyl 4-[(2S)-2-[2,5-dioxo-3-(4-phenoxyphenyl)imidazolidin-1-yl]-2-(5-fluoro-1-methyl-benzimidazol-2-yl)ethyl]piperidine-1-carboxylate (13D): At room temperature, triethylamine (0.5 ml, 3.6 mmol) was added to **13C** (0.57 g, 0.90 mmol) in dist.THF (15 ml) under a N_2 atmosphere. After stirring for 5 min, to the mixture, 4-nitrophenyl choloformate (0.36 g, 1.8 mmol) was added at ambient temperature and stirred for 15 h under the same conditions. The reaction mixture was quenched with sat.$NaHCO_3$ and extracted with EtOAc. The combined extracts were washed H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure. To the residue in THF (15 ml), TBAF (1M THF solution) (3.6 ml, 3.6 mmol) was added at ambient temperature and stirred for 3 h under the same conditions. The reaction mixture was quenched with sat.NH_4Cl and extracted with EtOAc. The combined extracts were washed with sat.$NaHCO_3$, H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give **13D** in 51 % yield. Rf=0.51 (*n*-Hexane/EtOAc=1/2), ^1H NMR (600 MHz, $CDCl_3$): δ 7.51-7.50 (m, 3H), 7.36-7.31 (m, 7H), 7.25-7.22 (m, 1H), 7.12-7.10 (m, 1H), 7.07-7.04 (m, 3H), 6.98-6.97 (m, 2H), 5.60-5.58 (m, 1H), 5.12 (s, 2H), 4.33 (s, 2H), 4.22 (br, 2H), 3.79 (s, 3H), 2.76 (m, 3H), 2.39 (m,

1H), 1.97-1.74 (m, 3H), 1.29 (m, 2H). HRMS (ESI/TOF) m/z: $[M + H]^+$ Calcd for $C_{38}H_{37}F_1N_5O_5$ 662.27787 ; Found 662.27818.

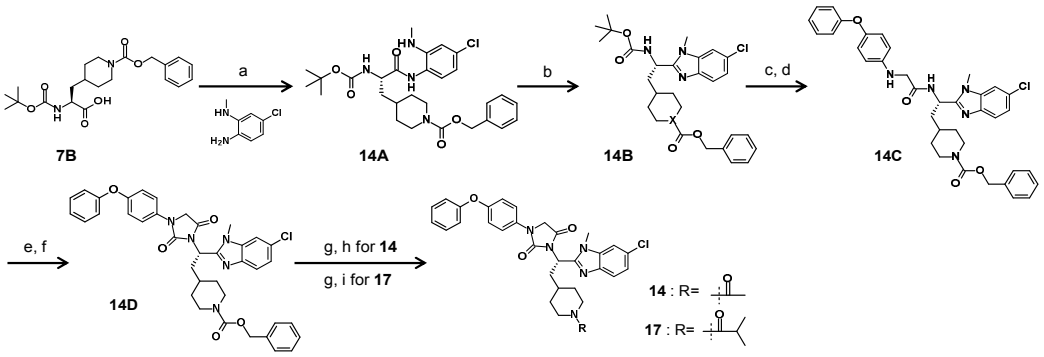
3-[(1S)-2-(1-Acetyl-4-piperidyl)-1-(5-fluoro-1-methyl-benzimidazol-2-yl)ethyl]-1-(4-phenoxyphenyl)imidazolidine-2,4-dione (13): To **13D** (290 mg, 0.44 mmol) and 10 % Pd/C (80 mg), was added dist.MeOH (18 ml) and hydrogenated under a hydrogen atmosphere for 3 h at ambient temperature. The solution was filtered and concentrated to provide a white solid (3-[(1S)-1-(5-fluoro-1-methyl-benzimidazol-2-yl)-2-(4-piperidyl)ethyl]-1-(4-phenoxyphenyl) imidazolidine-2,4-dione) in 91 % yield. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.52-7.33 (m, 3H), 7.32-7.26 (m, 2H), 7.13-7.10 (m, 1H), 7.11 (t, J=7.4 Hz, 1H), 7.04 (d, J=9.0 Hz, 3H), 6.97 (d, J=8.6 Hz, 2H), 5.63-5.61 (m, 1H), 4.34 (s, 2H), 3.80 (s, 3H), 3.49 (s, 2H), 3.08 (t, J=11.6 Hz, 2H), 2.86-2.80 (m, 1H), 2.58 (t, J=11.7 Hz, 2H), 2.40-2.37 (m, 1H), 1.96-1.94 (m, 1H), 1.75-1.73 (m, 1H), 1.46 (br, 1H), 1.32-1.12 (m, 2H). To a solution of (3-[(1S)-1-(5-fluoro-1-methyl-benzimidazol-2-yl)-2-(4-piperidyl)ethyl]-1-(4-phenoxyphenyl) imidazolidine-2,4-dione) (94 mg, 0.18 mmol) in CH_2Cl_2 (2 ml), triethylamine (62 µl, 0.45 mmol) and acetic anhydride (33 µl, 0.36 mmol) were added at ambient temperature and stirred for 3 h. The reaction mixture was quenched with sat. NH_4Cl and extracted with EtOAc. The combined extracts were washed with H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give **13** in 72% yield. Rf=0.78 (EtOAc/MeOH=9/1), ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.51 (dd, J=7.8, 4.2 Hz, 3H), 7.33 (t, J=7.8 Hz, 2H), 7.24 (dd, J=9.0, 4.8 Hz, 1H), 7.11 (t, J=7.8 Hz, 1H), 7.06 (brt, J=9.6 Hz, 1H), 7.04 (d, J=9.0 Hz, 2H), 6.98 (d, J=8.4 Hz, 2H), 5.59 (td, J=10.8, 6.0 Hz, 1H), 4.62 (t, J=14.4 Hz, 1H), 4.34 (d, J=7.2 Hz, 2H), 3.81 (m, 1H), 3.79 (s, 3H), 3.02 (td, J=12.0, 6.0 Hz, 1H), 2.89 (m, 0.5H), 2.69 (m, 0.5H), 2.58-2.50 (m, 1.5H), 2.34 (m, 0.5H), 2.08 (s, 3H), 2.01 (m, 1H), 1.08 (m, 1H), 1.61 (brs, 1H), 1.33-1.18 (m, 2H)., ^{13}C NMR (600 MHz, $CDCl_3$): δ 168.8, 167.7, 167.6, 160.2, 158.6, 157.1, 154.2, 153.4, 151.6, 151.5, 142.4, 142.3, 132.4, 129.9, 123.5, 120.2, 119.8, 118.7, 111.7, 109.7, 106.2, 49.8, 46.4, 45.5, 41.5, 36.1, 33.0, 32.8, 32.1, 31.8, 31.0, 30.3, 21.5., HRMS (ESI/TOF) m/z: $[M + H]^+$ Calcd for $C_{32}H_{33}F_1N_5O_4$ 570.25166 ; Found 570.25301.

3-[(1S)-1-(5-Fluoro-1-methyl-benzimidazol-2-yl)-2-[1-(2-methylpropanoyl)-4-piperidyl] ethyl]-1-(4-phenoxyphenyl)imidazolidine-2,4-dione (16): To a solution of (3-[(1S)-1-(5-fluoro-1-methyl-benzimidazol-2-yl)-2-(4-piperidyl)ethyl]-1-(4-phenoxyphenyl) imidazolidine-2,4-dione) (see synthetic procedure for **13**) (43 mg, 0.082 mmol) in CH_2Cl_2 (2 ml), triethylamine (14 µl, 0.098 mmol) and isobutylic anhydride (15 µl, 0.09 mmol) were added

at ambient temperature and stirred for 3 h. The reaction mixture was quenched with sat. NH_4Cl and extracted with EtOAc. The combined extracts were washed with H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give **16** in 44 % yield. Rf=0.25 (*n*-Hexane/EtOAc=1/3), ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.51 (d, J=6.0 Hz, 3H), 7.34 (t, J=7.8 Hz, 2H), 7.24 (dd, J=7.8, 4.2 Hz, 1H), 7.11 (t, J=7.8 Hz, 1H), 7.06 (brt, J=10.8 Hz, 1H), 7.04 (d, J=9.0 Hz, 2H), 6.98 (d, J=8.4 Hz, 2H), 5.60 (m, 1H), 4.66 (t, J=15.0 Hz, 1H), 4.34 (d, J=6.0 Hz, 2H), 3.95 (brd, J=12.6 Hz, 1H), 3.79 (s, 3H), 3.01 (m, 1H), 2.90 (m, 0.5H), 2.79 (quint., J=6.6 Hz, 1H), 2.69 (m, 0.5H), 2.58-2.51 (m, 1.5H), 2.33 (m, 0.5H), 2.02 (m, 1H), 1.81 (m, 1H), 1.64 (m, 1H), 1.31-1.18 (m, 2H), 1.11 (dd, J=12.6, 6.0 Hz, 6H)., ^{13}C NMR (600 MHz, $CDCl_3$): δ 175.3, 167.6, 166.3, 160.2, 158.6, 157.1, 154.2, 153.4, 151.6, 142.4, 132.4, 129.9, 123.5, 120.2, 119.8, 118.7, 111.6, 109.7, 106.1, 49.8, 45.5, 41.8, 36.1, 33.2, 33.0, 32.5, 31.9, 31.1, 30.3, 30.1, 19.6, 19.3., HRMS (ESI/TOF) m/z: $[M + H]^+$ Calcd for $C_{34}H_{37}F_1N_5O_4$ 598.28296 ; Found 598.28257.

7. Experimental procedure and analytical data of analogues 14 and 17

Scheme S4. Synthesis of Analogues 14 and 17[a]



[a]Reagents and conditions: (a) 4-Chloro-N2-methyl-benzene-1,2-diamine, HOAt, WSCI.HCl, DMF, 15 h, -20 °C -> rt; (b) CH_3COOH, 2 h, 80 °C; (c) 4 M HCl/dioxane, 1 h, rt; (d) 2-(4-Phenoxyanilino) acetic acid hydrochloride, HOAt, WSCI.HCl, 15 h, 0 °C -> rt; (e) 4-Nitrophenyl chloroformate, Et_3N, THF, 15 h, rt; (f) 1 M TBAF, THF, 3 h, rt; (g) 30 % HBr/CH_3COOH, 5 h, rt; (h) Acetic anhydride, DMF, Et_3N, 3 h, rt: (i) Isobutyric anhydride, DMF, Et_3N, 3 h, rt:

Benzyl 4-[(2S)-2-{[(tert-butoxy)carbonyl]amino}-2-{[4-chloro-2-(methylamino)phenyl] carbamoyl}ethyl]piperidine-1-carboxylate (14A): To a solution of **7B** (500 mg, 1.23 mmol) in DMF (5 ml), 4-chloro-N1-methyl-benzene-1,2-diamine[5] (193 mg, 1.23 mmol) was added at -20 °C. Subsequently, HOAt (184 mg, 1.35 mmol) and WSCI.HCl (354 mg, 1.85 mmol) were added to the former solution at -20 °C and stirred for 15 h at ambient temperature. The reaction mixture was quenched with sat.NH_4Cl and extracted with EtOAc. The combined extracts were washed with H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give **14A** in 74 % yield. Rf=0.50 (*n*-Hexane/EtOAc=1/1), ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.27 (s, 1H), 7.39-7.33 (m, 5H), 7.21 (d, J=6.6 Hz, 1H), 7.02 (d, J=7.8 Hz, 1H), 6.57 (brs, 2H), 5.29 (m, 1H), 5.08 (s, 2H), 4.09 (m, 1H), 4.02 (m, 2H), 2.83 (m, 2H), 2.72 (d, J=4.8 Hz, 3H), 1.72 (m, 2H), 1.57 (brs, 3H), 1.42 (s, 9H), 1.06 (m, 2H). HRMS (ESI/TOF) m/z: $[M + Na]^+$ Calcd for $C_{28}H_{37}Cl_1N_4O_5Na$ 567.23502 ; Found 567.23600.

Benzyl 4-[(2S)-2-{[(tert-butoxy)carbonyl]amino}-2-(6-chloro-1-methyl-1H-1,3-benzodiazol-2-yl)ethyl]piperidine-1-carboxylate (14B) : 14A (490 mg, 0.90 mmol) was dissolved in CH_3COOH (15 ml) at room temperature and stirred for 2 h at 80 °C. The reaction mixture was evaporated and quenched by addition of sat.$NaHCO_3$. The mixture was extracted with EtOAc, and organic layer was washed successively with H_2O, and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give **14B** in 88 % yield. Rf=0.45 (*n*-Hexane/EtOAc=1/1), ^{1}H NMR (600

MHz, DMSO-d_6): δ 7.71 (brs, 1H), 7.60 (d, J=9.0 Hz, 1H), 7.51 (d, J=8.4 Hz, 1H), 7.40-7.33 (m, 5H), 7.20 (d, J=8.4 Hz, 1H), 5.07 (s, 2H), 4.99 (m, 1H), 4.02 (m, 2H), 3.78 (s, 3H), 2.76 (m, 2H), 1.91 (m, 1H), 1.84 (m, 2H), 1.67 (m, 1H), 1.56 (m, 1H), 1.39-1.29 (brs, 9H), 1.08 (m, 2H). HRMS (ESI/TOF) m/z: $[M + H]^+$ Calcd for $C_{28}H_{36}Cl_1N_4O_4$ 527.24251 ; Found 527.24350.

Benzyl 4-[(2S)-2-(6-chloro-1-methyl-1H-1,3-benzodiazol-2-yl)-2-{2-[(4-phenoxyphenyl) amino]acetamido}ethyl]piperidine-1-carboxylate (14C): To an amorphous powder of **14B** (410 mg, 0.78 mmol), 4M HCl/dioxane (3.90 ml, 15.6 mmol) was added at room temperature and the mixture was stirred for 1 h at ambient temperature. Ether was added to the reaction mixture to yield the precipitate. The product was filtrated and dried over for 3 h *in vacuo*. The product in DMF (5 ml) was added to a solution of 2-[(4-phenoxyphenyl)amino]acetic acid hydrochloride[2] (218 mg, 0.78 mmol) and triethylamine (328 µl, 2.34 mmol) in DMF (3 ml) at 0 ^oC. Subsequently, HOAt (117 mg, 0.86 mmol) and WSCI.HCl (224 mg, 1.17 mmol) were added to the former solution at 0 ^oC and stirred for 15 h at ambient temperature. The reaction mixture was quenched with sat.NH_4Cl and extracted with EtOAc. The combined extracts were washed with H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give **14C** in 93 % yield. Rf=0.50 (*n*-Hexane/EtOAc=1/2), ^1H NMR (600 MHz, DMSO-d_6): δ 8.51 (d, J=9.0 Hz, 1H), 7.71 (d, J=2.4 Hz, 1H), 7.61 (d, J=7.8 Hz, 1H), 7.39-7.33 (m, 5H), 7.28 (t, J=8.4 Hz, 2H), 7.21 (dd, J=7.8, 1.8 Hz, 1H), 7.00 (t, J=7.8 Hz, 1H), 6.83 (d, J=9.0 Hz, 4H), 6.59 (d, J=9.0 Hz, 2H), 5.97 (t, J=6.0 Hz, 1H), 5.38 (m, 1H), 5.06 (s, 2H), 3.96 (m, 2H), 3.77-3.65 (m, 2H), 3.74 (s, 3H), 2.75-2.63 (m, 2H), 1.97 (m, 1H), 1.84 (m, 2H), 1.57 (m, 1H), 1.46 (m, 1H), 1.10 (q, J=7.8 Hz, 1H), 1.02 (q, J=11.4 Hz, 1H). HRMS (ESI/TOF) m/z: $[M + H]^+$ Calcd for $C_{37}H_{39}Cl_1N_5O_4$ 652.26906 ; Found 652.26876.

Benzyl 4-[(2S)-2-(6-chloro-1-methyl-1H-1,3-benzodiazol-2-yl)-2-[2,5-dioxo-3-(4-phenoxy phenyl)imidazolidin-1-yl]ethyl]piperidine-1-carboxylate (14D): At room temperature, triethylamine (504 µl, 3.60 mmol) was added to **14C** (470 mg, 0.72 mmol) in dist.THF (4 ml) under a N_2 atmosphere. After stirring for 5 min, to the mixture, 4-nitrophenyl choloformate (290 mg, 1.44 mmol) was added at ambient temperature and stirred for 15 h under the same conditions. The reaction mixture was quenched with sat.$NaHCO_3$ and extracted with EtOAc. The combined extracts were washed H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure. To the residue in THF (3 ml), TBAF (1M THF solution) (2.14 ml, 2.14 mmol) was added at ambient temperature and stirred for 3 h under the same conditions. The reaction mixture was quenched with sat.NH_4Cl and extracted with EtOAc. The combined extracts were

washed with sat.$NaHCO_3$, H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give **14D** in 74 % yield. Rf=0.80 (*n*-Hexane/EtOAc=1/4), ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.75 (d, J=1.8 Hz, 1H), 7.66 (dd, J=13.8, 9.0 Hz, 3H), 7.40-7.33 (m, 7H), 7.23 (dd, J=9.0, 1.8 Hz, 1H), 7.13 (t, J=7.2 Hz, 1H), 7.09 (d, J=9.0 Hz, 2H), 6.98 (d, J=7.8 Hz, 2H), 5.60 (m, 1H), 5.08 (s, 2H), 4.60 (m, 2H), 4.02 (m, 2H), 3.71 (s, 3H), 2.84 (m, 1H), 2.75 (m, 1H), 2.52 (m, 1H), 2.31 (m, 1H), 1.92 (m, 1H), 1.70 (m, 1H), 1.63 (m, 1H), 1.18 (q, J=12.0 Hz, 1H), 1.12 (q, J=11.4 Hz, 1H). HRMS (ESI/TOF) m/z: $[M + H]^+$ Calcd for $C_{38}H_{37}Cl_1N_5O_5$ 678.24832 ; Found 678.24874.

3-[(1S)-2-(1-Acetyl-4-piperidyl)-1-(6-chloro-1-methyl-benzimidazol-2-yl)ethyl]-1-(4-phenoxyphenyl)imidazolidine-2,4-dione (14): To an amorphous powder of **14D** (160 mg, 0.24 mmol), 30% HBr/CH_3COOH (471 µl, 2.40 mmol) was added at room temperature and the mixture was stirred for 5 h at ambient temperature. Ether was added to the reaction mixture to yield the precipitate. The product was filtrated and dried over for 3 h *in vacuo*. To the product in DMF (3 ml), acetic anhydride (24.5 µl, 0.26 mmol) and triethylamine (67.2 µl, 0.48 mmol) ware added to the former solution at room temperature and stirred for 3 h at ambient temperature. The reaction mixture was quenched with sat.NH_4Cl and extracted with EtOAc. The combined extracts were washed with H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give **14** in 87% yield. Rf=0.35 (EtOAc), ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.76 (brd, J=5.4 Hz, 1H), 7.51 (dd, J=7.8, 4.2 Hz, 2H), 7.35-7.32 (m, 3H), 7.24 (m, 1H), 7.12 (t, J=7.8 Hz, 1H), 7.04 (d, J=9.0 Hz, 2H), 6.98 (d, J=8.4 Hz, 2H), 5.60 (m, 1H), 4.62 (brt, J=13.8 Hz, 1H), 4.34 (d, J=7.2 Hz, 2H), 3.82 (brd, J=12.6 Hz, 1H), 3.77 (s, 3H), 3.03 (m, 1H), 2.88 (m, 0.5H), 2.70 (m, 0.5H), 2.54 (m, 1.5H), 2.35 (m, 0.5H), 2.08 (s, 3H), 2.02 (brt, J=13.2 Hz, 1H), 1.80 (m, 1H), 1.63 (m, 1H), 1.35-1.17 (m, 2H)., ^{13}C NMR (600 MHz, $CDCl_3$): δ 168.8, 167.7, 167.5, 157.0, 154.2, 153.3, 151.0, 150.8, 140.3, 136.5, 132.4, 129.9, 129.2, 123.5, 123.3, 121.2, 120.2, 119.8, 118.7, 109.6, 49.8, 46.4, 45.4, 41.5, 36.1, 32.9, 32.8, 32.1, 31.8, 31.0, 30.3, 21.5., HRMS (ESI/TOF) m/z: $[M + Na]^+$ Calcd for $C_{32}H_{32}Cl_1N_5O_4Na$ 608.20405 ; Found 608.20659.

3-[(1S)-1-(6-Chloro-1-methyl-benzimidazol-2-yl)-2-[1-(2-methylpropanoyl)-4-piperidyl]ethyl]-1-(4-phenoxyphenyl)imidazolidine-2,4-dione (17): To an amorphous powder of **14D** (60 mg, 0.088 mmol), 30% HBr/CH_3COOH (345 µl, 1.76 mmol) was added at room temperature and the mixture was stirred for 5 h at ambient temperature. Ether was added to the reaction mixture to yield the precipitate. The product was filtrated and dried over for 3h *in vacuo*. To the product in DMF (3 ml), isobutyric anhydride (16.1 µl, 0.097 mmol) and

triethylamine (24.6 μl, 0.176 mmol) ware added to the former solution at room temperature and stirred for 3 h at ambient temperature. The reaction mixture was quenched with sat.NH_4Cl and extracted with EtOAc. The combined extracts were washed with H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give **17** in 99 % yield. Rf=0.55 (*n*-Hexane/EtOAc = 1/4), ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.74 (dd, J=9.0, 4.2 Hz, 1H), 7.51 (brd, J=7.2 Hz, 2H), 7.35-7.32 (m, 3H), 7.23 (brd, J=7.8 Hz, 1H), 7.11 (t, J=8.4 Hz, 1H), 7.04 (d, J=9.0 Hz, 2H), 6.98 (d, J=9.0 Hz, 2H), 5.60 (m, 1H), 4.66 (brt, J=15.6 Hz, 1H), 4.34 (d, J=5.4 Hz, 2H), 3.95 (brd, J=13.2 Hz, 1H), 3.76 (s, 3H), 3.00 (m, 1H), 2.88 (m, 0.5H), 2.79 (quint., J=6.6 Hz, 1H), 2.70 (m, 0.5H), 2.52 (m, 1.5H), 2.35 (m, 0.5H), 2.02 (brt, J=13.2 Hz, 1H), 1.81 (m, 1H), 1.63 (m, 1H), 1.25 (m, 1H), 1.20 (m, 1H), 1.11 (dd, J=12.6, 6.0 Hz, 6H)., ^{13}C NMR (600 MHz, $CDCl_3$): δ 175.3, 167.7, 167.6, 157.1, 154.2, 153.3, 151.1, 151.0, 140.6, 136.5, 132.4, 129.9, 129.1, 123.5, 123.2, 121.3, 120.2, 119.8, 118.7, 109.5, 49.8, 45.5, 41.8, 36.1, 33.2, 33.0, 32.4, 31.9, 31.1, 30.3, 30.1, 19.6, 19.3., HRMS (ESI/TOF) m/z: $[M + H]^+$ Calcd for $C_{34}H_{37}Cl_1N_5O_4$ 614.25341 ; Found 614.25257.

8. Analytical data of 6 and 1

3-[(1S)-2-Cyclohexyl-1-(1,6-dimethyl-1H-1,3-benzodiazol-2-yl)ethyl]-1-(4-phenoxyphenyl)imidazolidine-2,4-dione (6): The ^{1}H-NMR and HRMS data of **6** posted the data reported in the reference 6. ^{13}C NMR (600 MHz, $CDCl_3$): δ 167.7, 157.2, 153.9, 153.7, 150.4, 140.2, 136.1, 133.0, 132.8, 129.8, 123.8, 123.4, 120.2, 119.9, 119.8, 118.6, 109.2, 49.7, 46.0, 37.0, 34.4, 33.5, 32.4, 30.0, 26.4, 26.1, 21.9. ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.65 (d, J = 9.0 Hz, 2H), 7.52 (d, J = 8.4 Hz, 1H), 7.39 (t, J = 7.8 Hz, 2H), 7.33 (s, 1H), 7.13 (t, J = 7.8 Hz, 1H), 7.09 (d, J = 9.6 Hz, 2H), 7.03 (d, J = 9.0 Hz, 1H), 6.98 (d, J = 7.2 Hz, 2H), 5.57–5.55 (m, 1H), 4.61 (d, J = 3 Hz, 2H), 3.68 (s, 3H), 2.48 (m, 1H), 2.45 (s, 3H), 2.26 (m, 1H), 1.97 (m, 1H), 1.70 (m, 3H), 1.63 (m, 1H), 1.39 (m, 1H), 1.25–1.19 (m, 3H), 1.06 (m, 1H), 0.97 (m, 1H). HRMS (ESI/TOF) m/z: $[M + Na]^+$ Calcd for $C_{32}H_{34}N_4O_3Na$ 545.25286 ; Found 545.25300.

4-[4-({6-[1-tert-Butyl-5-(4-fluorophenyl)-1H-pyrazole-4-amido]-1H-indazol-1-yl}methyl)piperidin-1-yl]benzoic acid (1): 1 was prepared as the reference compound according to the method of reference 7. ^{1}H NMR (600 MHz, $CDCl_3$): δ 12.25 (brs, 1H), 9.78 (s, 1H), 8.14 (s, 1H), 7.97 (d, J=5.4 Hz, 2H), 7.74 (d, J=9.0 Hz, 2H), 7.64 (d, J=8.4 Hz, 1H), 7.46 (dd, J=8.4, 6.6 Hz, 2H), 7.30 (t, J=9.0 Hz, 2H), 7.19 (d, J=9.0 Hz, 1H), 6.92 (d, J=9.0 Hz, 2H), 4.21 (d, J=7.2 Hz, 2H), 3.88 (d, J=13.2 Hz, 2H), 2.76 (t, J=12.0 Hz, 2H), 2.15 (m, 1H), 1.50 (brd, J=10.8 Hz, 2H), 1.40 (s, 9H), 1.30 (brq, J=12.0 Hz, 2H)., ^{13}C NMR (600 MHz, $CDCl_3$): δ 167.3, 161.5, 160.9, 153.5, 142.7, 139.9, 137.6, 136.5, 133.0, 132.9, 132.5, 130.9, 128.3, 120.8, 119.7, 118.6,

118.4, 115.0, 114.9, 114.7, 113.3, 98.8, 62.1, 53.0, 46.7, 40.0, 36.6, 30.7, 28.7., HRMS (ESI/TOF) m/z: $[M + Na]^+$ Calcd for $C_{34}H_{35}F_1N_6O_3Na$ 617.26524 ; Found 617.26415.

9. Purity determination (1 and 6-17)

Analytical HPLC analyses were performed using the following conditions:
Column name and size; Tsk-GEL, ODS-120T 4.6x150mm. Solvent A: H_2O/TFA (100/0.1). Solvent B: CH_3CN/TFA (100/0.1). Flow rate: 1.0 ml/min. Gradient time: 30 min; 0-30 min 30 % B → 80 % B . Monitored by UV at 254nm.

Purity of compounds 1 and 6-17

Cpd No.	Purity (%)	Retention time (min)
1*	95.4	19.219
6**	98.4	25.315
7	99.4	13.177
8	96.3	15.395
9	99.3	16.986
10	99.4	19.723
11	99.0	21.836
12	99.8	15.231
13	99.5	15.969
14	98.8	18.002
15	99.5	18.338
16	99.3	18.892
17	98.8	20.946

*: Kitamura, S. et al., Jpn. Kokai Tokkyo Koho. JP 2008273847. 13 Nov 2008

: Teno, N. *et al*., *Bioorg.Med.Chem.*, **2017, 25, 1787-1794.

10. Antagonistic rate of 6-17 at each concentration in luciferase reporter gene assay

Table S1

Cpds	Antagonistic rate (%)
6*	97.6 (4 μM)
7	100 (20 μM)
8	100 (20 μM)
9	100 (0.8 μM)
10	100 (1.3×10^{-3} μM)
11	100 (1.3×10^{-3} μM)
12	100 (0.16 μM)
13	100 (0.8 μM)
14	100 (0.8 μM)
15	100 (1.3×10^{-3} μM)
16	100 (6.4×10^{-3} μM)
17	100 (6.4×10^{-3} μM)

*: Reference 6

11. Biology

Plasmids. To prepare human VDR expression plasmids, the corresponding cDNAs were amplified by PCR with KOD FX Neo (Toyobo) using a set of primers: 5'-TGCTCCTTCAGGGATGGAGGCAAT-3' and 5'-CCACAGGCTGTCCTAGTCAGGAGATCT-3'. Human intestine cDNA was used as a template and subcloned into pBlueScript II cloning vector (Agilent Technologies), and then inserted into the BamHI and XhoI sites of pcDNA3.1/Hygro vector (Invitrogen). To prepare 3x hCYP3A4-ER6-pGL4.10 (VDRE-Luc), a set of oligonucleotides: 5'-TCGAATATGAACTCAAAGGAGGTCAGTGATATGAACTCAAAGGAGGTCAGTGATA TGAACTCAAAGGAGGTCAGTG-3' and 5'-AGCTCACTGACCTCCTTTGAGTTCATATCACTGACCTCCTTTGAGTTCATATCACTG ACCTCCTTTGAGTTCATAT-3' were annealed and inserted into the XhoI and HindIII sites of pGL4.10. The sequences and orientations of these plasmids were confirmed by direct sequencing. cDNAs encoding full-length hPPARα and δ were PCR-cloned and inserted into mammalian expression vector pcDNA3.1 (Invitrogen) according to previous report in which hPPARγ expression vector was prepared.[8]

Reporter Gene Assays and Co-Activator Recruitment Assay. Reporter gene assays for FXR, RXR, PPARα, δ, γ, LXRα, β, RARα and TGR5 were performed as previously described.[6, 9, 10] The luciferase assay for FXR would be summarized as follows; Hep3B cells were seeded onto 96-well plates. After 16-24 hr, cells were transfected with hFXR and hRXRα expression vectors, FXR response element-driven luciferase vector and β-galactosidase control vector. After five hours, the cells were treated with test compounds (10^{-12} – 10^{-4} M) in the presence of 5μM of chenodeoxycholic acid (CDCA). The cells were then lysed, followed by determination of the luciferase activity. The firefly luciferase activity was normalized to the β-galactosidase activity for each well. To obtain the IC_{50} values of each compound, the regression formula was presented by using ImageJ, software capable of generating a 4-parameter logistic curve fit. And as calculated CDCA treatment alone for 100%, IC_{50} values were determined. For cell-based luciferase assay of VDR, HEK293T cells were plated in 96-well poly-D-lysine coated plate, and

were transfected with hVDR and hRXRα expression plasmids, VDRE-Luc reporter plasmid and *Renilla* luciferase vector. After 24 hr, the cells were exposed to test compounds or DMSO (N.C.) in DMEM supplemented with 0.5% delipidated serum for 24 hr. Luciferase activities were determined in cell lysates. Firefly luciferase activity was normalized to either that of *Renilla* luciferase for each well.

To evaluate the binding ability of FXR antagonist candidates, LanthaScreen® TR-FRET FXR Coactivator Assay Kit (Thermo Fisher Scientific Inc.) was used as the previous study.[6] Briefly, different concentrations of test compounds ($4x10^{-11}$ – $4x10^{-4}$ M) and 0.5μM of GW4064 which is synthesis FXR agonist were mixed with FXR-LBD-Glutathione S-transferase fusion protein, Fluorecein-SRC2-2 coactivator peptide and Lantha-screen Tb-anti GST antibody in 384-well black plate. EnVision® Multilabel Reader (PerkinElmer) was used for detection of the TR-FRET signal. The IC_{50} values of each compound were determined by constructing a dose-response curve calculated GW4064 treatment alone for 100% likewise reporter gene assays.

Real-time RT-PCR assay. For FXR target gene expression analysis, Huh-7 cells were subcultured in 6-well plates and exposed to analogue **10** in the presence of CDCA (10 μM) or GW4064 (1 μM) for 24 hr. To evaluate the effect of analogue **10** on pregnane X receptor (PXR) target gene expression, HepG2 cells were treated with both analogue **10** and Rifampicin (10 μM) for 48 hr. Total RNA was isolated, and then reversely transcribed into cDNA. mRNA levels of bile salt export pump (BSEP), small heterodimer partner (SHP), organic solute transporter alpha (OSTα), cytochrome P450 subfamily (CYP) 3A4 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were quantified by real-time RT-PCR using specific primers. mRNA levels were all normalized to GAPDH mRNA. All values were reported as means ± S.D. of triple measurements of each cDNA sample. The specific primers for human BSEP, SHP, CYP3A4 and GAPDH have been reported previously.[6, 11-13] The primer sequences for human OSTα are the following: forward: ACCCTGGTGGGCCTGTTT; reverse: CAGAAATGTCTGCTGGGTCATAGA.

Cell Culture. Mouse adipocytic 3T3-L1 cells were purchased from Human Science Research Resources Bank, and grown in DMEM containing 10 % fetal bovine serum. Preadipocytes were differentiated into adipocytes for 2 days in DMEM containing 10 μg/ml insulin (Sigma), 1 μM dexamethasone (Sigma), and 0.5 mM 3-isobutyl-1-methylxanthine (Nacalai Tesque). After 2 days, the medium was replaced with DMEM containing insulin (10 μg/ml) alone and changed every 2 days. Oil Red O staining of the lipids in the cells was carried out as described previously.[14]

Cell Toxicity Assay. The cell toxicity of each compound to Hep3B cells was determined by the MTT assay which was performed in accordance with the previous report.[15] 3T3-L1 preadipocytes were seeded in 96-well culture plates and then grown in DMEM containing various concentrations of analogue **10** (0-10 μM) for 6 days. The medium with analogue **10** was changed every 2 days. Thereafter, cell toxicity was measured using a Cell Counting Kit-8 (Dojindo).

Measurement of Intracellular Triacylglycerol Level. 3T3-L1 preadipocytes were differentiated into adipocytes for 6 days as described above. The intracellular triacylglycerol level was measured by the use of a WAKO LabAssay Triglyceride Kit (Wako Pure Chemical). Protein concentrations were measured with a Pierce BCA Protein Assay Reagent (Thermo Fisher Scientific).

Statistical Analysis. All data were presented as means ± standard deviation (SD). To determine significant differences between 2 groups, comparisons were made using the Student's t tests. When compared multiple groups, one-way ANOVA and a Tukey's post-hoc analysis were performed. The significance of differences was defined at $p<0.05$.

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