Supporting Information (SI Appendix)

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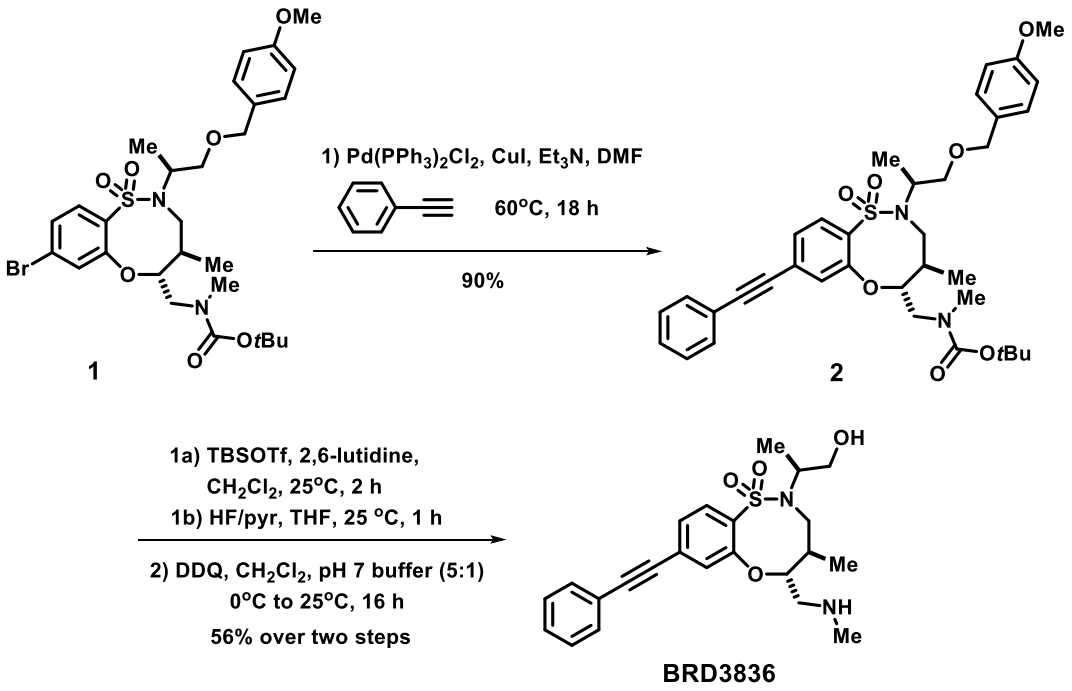
Cell lines and reagents. HeLa cell lines were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FBS (Corning), 1X GlutaMAX, 55 μM β-mercaptoethanol, 2.5 mM NaOH, and 100 U/mL penicillin/streptomycin. *Atg5*$^{+/+}$ and *Atg5*$^{-/-}$ MEFs (gift from Noboru Mizushima) (1) were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin/streptomycin, 2 mM L-glutamine, and 1% non-essential amino acids in a 37°C, 5% CO_2 humidified incubator. Human embryonic stem cells (hESCs) (WIBR3 cells) and Niemann-Pick type C1 disease (NPC1) patient-specific human induced pluripotent stem cells (hiPSCs) (WIBR-IPS-NPC1 cells) were cultured as described previously (2). Briefly, hESCs and hiPSCs were maintained on mouse embryonic fibroblast (MEF) feeder layers in hESC medium [DMEM/F12 supplemented with 15% fetal bovine serum (FBS, HyClone), 5% KnockOut Serum Replacement, 1 mM glutamine, 100 U/mL penicillin/streptomycin, 1% nonessential amino acids (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma), and 4 ng/mL FGF2 (R&D Systems)]. Cell lines were passaged every 5 to 7 days with 1.5 mg/mL collagenase type IV. Reagents used for cell culture were from Life Technologies unless otherwise stated. EGFP-HDQ74 construct (mutant huntingtin exon 1 with 74 polyglutamine repeats) was a gift from David Rubinsztein and was used as previously described (3). Reagents were obtained from the following suppliers: PI-103 (Calbiochem), Torin1 (Tocris Bioscience), bafilomycin A1 (LC Laboratories), chloroquine, carbamazepine, pepstatin A, rottlerin, trifluoperazine, and chloramphenicol (Sigma-Aldrich), E64d (Santa Cruz Biotechnology), dexamethasone (Cayman Chemical), VX-765 (Selleck Chemicals). DOS compounds were obtained from the Broad Institute Compound Management Group in 8-point twofold dilution series from a top concentration of 10 μM and were evaluated for ≥ 90% purity by high-performance liquid chromatography-mass spectrometry (HPLC-MS).

Chemical Synthesis. All chemicals and solvents were purchased from Sigma-Aldrich, Acros Organics, Alfa Aesar, ArkPharm, TCI America, ChemBridge, or Matrix Scientific. Chemicals were used without further purification. Reactions were monitored by thin-layer chromatography (TLC) using E. Merck silica gel 60 F254 pre-coated plates (0.25 mm) or by tandem liquid chromatography-mass spectrometry (LC-MS) using a Waters 2795 separations module and 3100 mass detector. Visualization for TLC was achieved with UV light and/or an iodine chamber. NMR spectra were measured on a Bruker Ultrashield 300 (300 MHz ^{1}H, 75 MHz ^{13}C) or a Bruker Ultrashield 400 (400 MHz ^{1}H, 100 MHz ^{13}C). Proton chemical shifts are reported in ppm (δ) referenced to the NMR solvent. Data are reported as follows: chemical shifts, multiplicity (s = singlet, d = doublet, t = triplet, dd = doublet of doublets, q =quartet, dt = doublet of triplets, ddd= doublet of doublet of doublets, m = multiplet), coupling constant(s) in Hz, integration. All NMR data were collected at 25°C. Infrared spectra were obtained on a Thermo Scientific Nicolet IR100 FT-IR spectrometer and are reported in cm^{-1}. Compounds were dried on polytetrafluoroethylene (PTFE) plates for infrared analysis. Optical rotation was measured on a Rudolph Research Analytical Autopol IV automatic polarimeter. Flash chromatography purifications were performed using a CombiFlash Rf system (Teledyne ISCO) with pre-packed 40-60 μm Silica Gel (60 Å mesh) columns. High resolution LC-MS (HRMS) was performed on an Agilent 1290 Infinity separations module and 6230 time-of-flight (TOF) mass detector operating in ESI+ mode. Synthetic intermediates and final compounds were characterized by ^{1}H NMR, IR, ^{13}C NMR, and HRMS. BRD5631, BRD7424, BRD34009, BRD3836, and BRD2716 were additionally characterized by optical rotation. Analogs of BRD5631 were characterized by ^{1}H NMR, IR, and HRMS.

Synthetic Procedures.

Scheme 1. Synthesis of BRD3836.

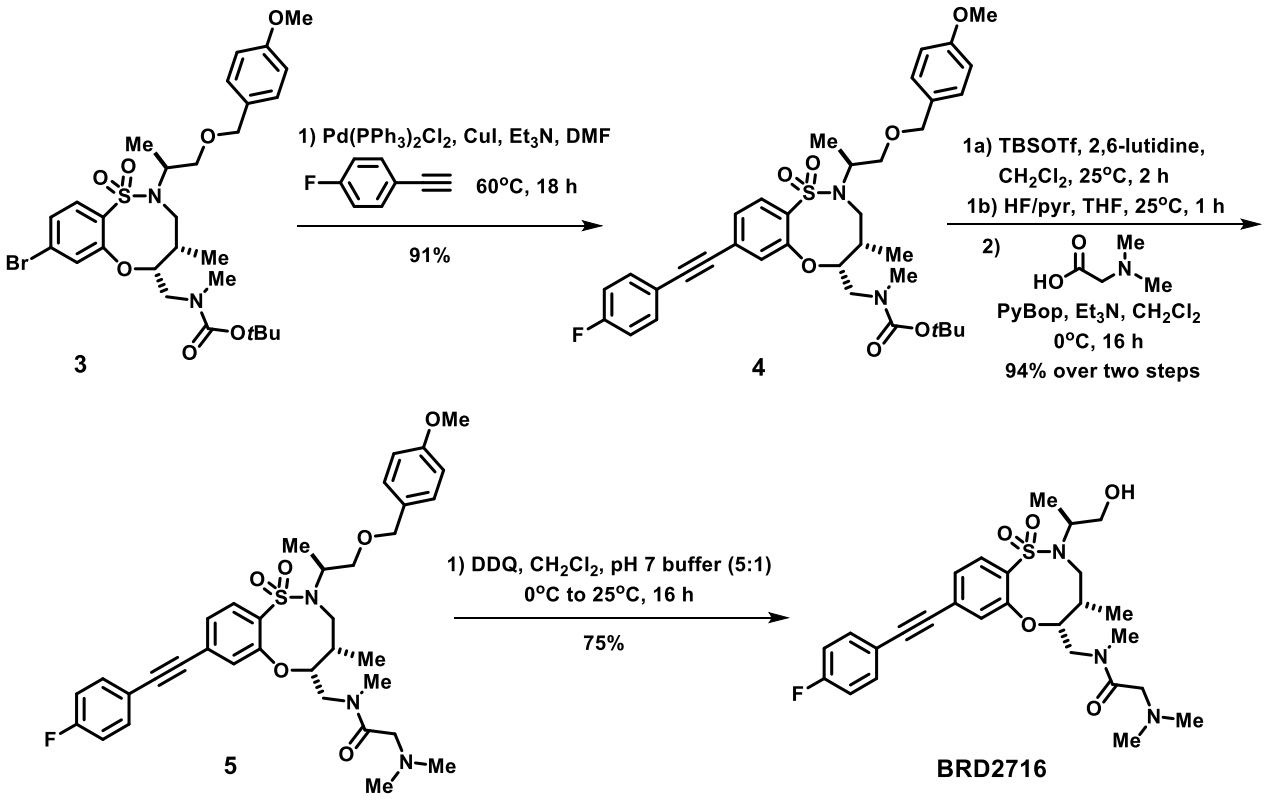


Procedure: Sultam **1** was prepared according to literature procedure (4). Sultam **1** (100 mg, 0.159 mmol) was suspended in DMF (2.7 mL, 0.06 M) and Et_3N (0.56 mL, 3.98 mmol). Phenylacetylene (87 µl, 0.797 mmol) was added at room temperature, followed by $Pd(PPh_3)Cl_2$ (28.0 mg, 0.040 mmol) and finally CuI (45.5 mg, 0.239 mmol). The reaction vessel was purged and backfilled with N_2 (2x) and was then heated to 60°C for 18 h. The reaction mixture was cooled to room temperature, quenched with water, extracted with ethyl acetate, dried over sodium sulfate, and condensed *in vacuo*. The resulting product was purified by flash chromatography (Isco, silica 12 g, 0-35% EtOAc in Hexanes, 28 min) to give **2** as a light brown oil (93 mg, 90%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.85 (d, *J* = 8.1 Hz, 1H), 7.53 (s, 2H), 7.37 (s, 3H), 7.31 (d, *J* = 8.0 Hz, 1H), 7.13 (s, 1H), 7.07 (s, 2H), 6.84 (d, *J* = 7.9 Hz, 2H), 4.41 (s, 1H), 4.23 (d, *J* = 25.2 Hz, 2H), 4.15 – 3.92 (m, 1H), 3.77 (s, 3H), 3.71 – 3.54 (m, 3H), 3.54 – 3.35 (m, 1H), 3.15 – 2.81 (m, 2H), 2.73 (s, 2H), 2.16 (s, 1H), 1.75 – 1.54 (m, 1H), 1.50 (s, 9H), 1.32 (s, 3H), 0.93 (d, *J* = 5.8 Hz, 3H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 159.17, 155.90, 131.71, 129.95, 129.24, 128.86, 128.43, 126.79, 126.41, 122.50, 113.69, 92.31, 87.82, 84.76, 79.92, 72.77, 72.41, 64.35, 60.38, 56.25, 55.22, 51.33, 36.52, 34.75, 30.65, 28.37, 21.04, 21.00, 19.12, 16.53, 15.48, 14.20, 13.70. **IR** (PTFE) ν_{max} = 3060, 2974, 2215, 1695, 1593, 1545, 1513, 1469, 1395, 1367, 1327, 1247, 1154, 1066, 1013, 938, 827, 759, 694, 637, 548, 513 cm^{-1}. **HRMS** $C_{36}H_{44}N_2O7_S$; calculated $(M+Na)^+$ = 671.2761; average observed $(M+Na)^+$ = 671.2772.

2,6-Lutidine (0.034 mL, 0.287 mmol) and TBSOTf (0.05 mL, 0.215 mmol) were added to a solution of diphenyl alkyne **2** (46.5 mg, 0.072 mmol) in DCM (1.42 mL, 0.05 M) at room temperature. The mixture was stirred until complete consumption of starting material was observed by LCMS (2 h). The reaction was quenched with saturated $NaHCO_3$ solution and extracted with DCM (2x). The combined organic extracts were dried over sodium sulfate, filtered, and concentrated to give the silyl carbamate. The crude oil was dissolved in THF (1.42 mL, 0.05 M), HF/pyridine (70% w/w, 9.22 µL, 0.072 mmol) was added, and the resulting mixture stirred for 1 h at room temperature. The reaction was quenched by dropwise addition of saturated $NaHCO_3$ solution and extracted with EtOAc. The combined organic extracts were dried over sodium sulfate, filtered, and concentrated to provide the secondary amine, which was used without further purification.

Crude amine (39.3 mg, 0.072 mmol) was suspended in DCM and pH 7 phosphate buffer (4:1, 3.6 mL, 0.02 M) and cooled to 0°C. DDQ (24.4 mg, 0.107 mmol) was added in one portion and the solution was allowed to slowly warm to room temperature and stirred overnight (16 h). The reaction was quenched by addition of saturated $NaHCO_3$ solution, extracted with DCM (3x), dried over sodium sulfate, filtered, and concentrated. The crude product was purified by flash chromatography (Isco, silica 4 g, 0-10% MeOH in DCM, 32 min) to give the final product, **BRD3836**, as a light yellow oil (17.1 mg, 56% over two steps). $[\alpha]_D^{20}$ = -30.4° (c 0.6, $CHCl_3$). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.92 (d, *J* = 7.6 Hz, 1H), 7.56 (s, 2H), 7.39 (s, 3H), 7.28 (s, 2H), 4.48 (s, 1H), 3.93 (dd, *J* = 27.3, 11.2 Hz, 2H), 3.72 – 3.50 (m, 2H), 3.44 (d, *J* = 13.7 Hz, 1H), 3.12 – 3.02 (m, 2H), 2.94 – 2.84 (m, 1H), 2.60 (s, 3H), 2.45 (s, 1H), 1.26 (d, *J* = 6.2 Hz, 3H), 0.98 (s, 3H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 134.82, 131.82, 131.70, 129.55, 129.03, 128.53, 128.46, 127.99, 127.12, 122.27, 99.98, 92.95, 87.31, 64.48, 57.73, 47.85, 29.70, 15.67, 14.99. **IR** (PTFE) ν_{max} = 3341, 2928, 1594, 1546, 1493, 1469, 1396, 1322, 1215, 1153, 1065, 1011, 978, 891, 832, 758, 692, 636, 548, 513 cm^{-1}. **HRMS** $C_{23}H_{28}N_2O_4S$; calculated $(M+H)^+$ = 429.1843; average observed $(M+H)^+$ = 429.1840.

Scheme 2. Synthesis of BRD2716.



Procedure: Sultam **3** was prepared according to literature procedure (4). Sultam **3** (500 mg, 0.797 mmol) was suspended in DMF (13.3 mL, 0.06 M) and Et_3N (4.44 mL, 31.9 mmol). 1-ethynyl-4-fluorobenzene (383 mg, 3.19 mmol) was added at room temperature, followed by $Pd(PPh_3)Cl_2$ (140 mg, 0.199 mmol) and finally CuI (228 mg, 1.195 mmol). The reaction vessel was purged and backfilled with N_2 (2x) and was then heated to 60°C for 18 h. The reaction mixture was cooled to room temperature, quenched with water, extracted with ethyl acetate, dried over sodium sulfate, and condensed *in vacuo*. The resulting product was purified by flash chromatography (Isco, silica 24 g, 0-40% EtOAc in Hexanes, 36 min) to give **4** as a brown foaming solid (484 mg, 91%).**^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.86 (d, *J* = 5.7 Hz, 1H), 7.52 (s, 2H), 7.36 – 7.30 (m, 1H), 7.20 (s, 1H), 7.07 (t, *J* = 8.4 Hz, 2H), 6.98 (s, 2H), 6.81 (d, *J* = 7.9 Hz, 2H), 4.48 (d, *J* = 67.0 Hz, 1H), 4.19 (q, *J* = 11.7 Hz, 2H), 4.11 (dd, *J* = 15.4, 9.7 Hz, 1H), 3.80 (d, *J* = 7.5 Hz, 2H), 3.76 (s, 3H), 3.65 (dd, *J* = 13.6, 5.4 Hz, 1H), 3.44 (s, 2H), 3.27 (s, 1H), 2.66 (s, 3H), 2.13 (s, 1H), 1.44 (s, 9H), 1.24 (s, 3H), 1.00 (d, *J* = 6.3 Hz, 3H). **^{13}C NMR** (100 MHz, $CDCl_3$) Reported as a mixture of rotamers. δ 164.11, 161.62, 159.24, 156.08, 137.74, 133.74,

133.65, 132.15, 132.05, 130.04, 129.72, 129.43, 128.56, 128.44, 128.06, 127.66, 127.13, 126.88, 118.53, 115.95, 115.73, 114.13, 113.74, 113.71, 87.33, 84.52, 84.13, 79.77, 72.74, 72.17, 64.35, 56.08, 55.26, 55.21, 49.68, 49.10, 48.34, 35.49, 34.64, 34.41, 33.87, 30.65, 28.39, 21.01, 19.12, 17.23, 13.70, 12.74. **IR** (PTFE) v_{max} = 3066, 2972, 2934, 2878, 1692, 1612, 1589, 1549, 1511, 1459, 1393, 1367, 1330, 1300, 1253, 1213, 1176, 1143, 1095, 1066, 1034, 982, 921, 873, 838, 769, 730, 624, 600, 569 cm^{-1}. **HRMS** $C_{36}H_{43}FN_2O_7S$; calculated $(M+Na)^+$ = 689.2667; average observed $(M+Na)^+$ = 689.2634.

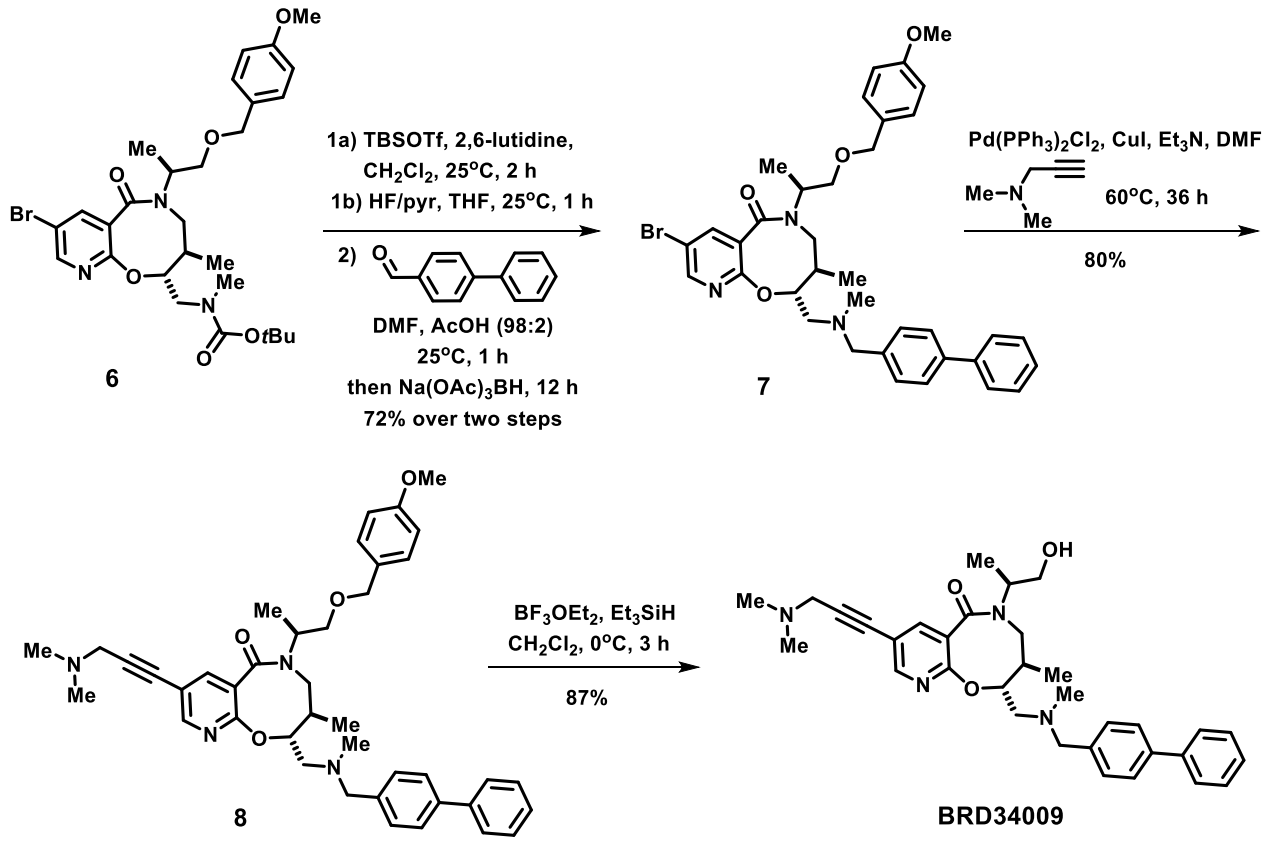
2,6-Lutidine (0.169 mL, 1.452 mmol) and TBSOTf (0.250 mL, 1.089 mmol) were added to a solution of 4-fluoro-diphenyl alkyne **4** (242 mg, 0.363 mmol) in DCM (7.25 mL, 0.05 M) at room temperature. The mixture was stirred until complete consumption of starting material was observed by LCMS (2 h). The reaction was quenched with saturated $NaHCO_3$ solution and extracted with DCM (2x). The combined organic extracts were dried over sodium sulfate, filtered, and concentrated to give the silyl carbamate. The crude oil was dissolved in THF (7.25 mL, 0.05 M), HF/pyridine (70% w/w, 0.047 mL, 0.363 mmol) was added, and the resulting mixture stirred for 1 h at room temperature. The reaction was quenched by dropwise addition of saturated $NaHCO_3$ solution and extracted with EtOAc. The combined organic extracts were dried over sodium sulfate, filtered, and concentrated to provide the secondary amine, which was used without further purification.

Crude amine (206 mg, 0.364 mmol) was suspended in DCM (7.25 mL, 0.05 M) and Et_3N (0.304 mL, 2.181 mmol) was added followed by 2-(dimethylamino)acetic acid (150 mg, 1.454 mmol) and PyBOP (378 mg, 0.727 mmol) at room temperature, and the resulting mixture was stirred overnight. The reaction was quenched with water and extracted with DCM (3x). The combined organic extracts were dried over sodium sulfate, filtered, and concentrated. The resulting yellow oil was taken up in Et_2O and the phosphoramide by-products removed by filtration. The solvent was removed *in vacuo* and the crude product was purified by flash chromatography (Isco, silica 24 g, 0-10% DCM in MeOH, 32 min) to give **5** as a yellow oil (223 mg, 94% over two steps). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.10 (s, 1H), 7.82 (dd, *J* = 23.6, 8.0 Hz, 1H), 7.59 – 7.50 (m, 2H), 7.46 (s, 1H), 7.33 (t, *J* = 11.0 Hz, 1H), 7.23 – 7.11 (m, 2H), 7.05 (t, *J* = 8.2 Hz, 2H), 6.82 (dd, *J* = 26.6, 8.2 Hz, 2H), 4.81 (d, *J* = 14.3 Hz, 1H), 4.42 – 4.24 (m, 2H), 4.18 – 4.04 (m, 3H), 3.74 (d, *J* = 14.3 Hz, 4H), 3.60 – 3.49 (m, 1H), 3.48 – 3.35 (m, 1H), 3.28 (d, *J* = 14.0 Hz, 1H), 3.07 (s, 3H), 3.01 (s, 3H), 2.79 (s, 2H), 2.33 (s, 1H), 1.81 (s, 3H), 1.03 (d, *J* = 6.5 Hz, 3H), 0.86 (d, *J* = 6.9 Hz, 3H). **^{13}C NMR** (100 MHz, $CDCl_3$) Reported as a mixture of rotamers. δ 165.95, 164.92, 164.21, 161.72, 159.30, 159.24, 153.35, 136.55, 133.99, 133.94, 133.91, 133.86, 129.78, 129.58, 129.55, 129.30, 129.25, 127.90, 127.59, 125.08, 118.25, 118.22, 115.98, 115.91, 115.75, 115.69, 113.78, 113.74, 92.52, 86.79, 83.24, 72.75, 72.66, 71.97, 57.99, 55.76, 55.26, 55.23, 47.54, 47.36, 46.70, 46.41, 46.36, 44.70, 44.05, 35.27, 34.26, 33.88, 26.40, 26.32, 17.01, 16.83, 14.48, 13.05, 8.72. **IR** (PTFE) v_{max} =3642, 3209, 3074, 2973, 2875, 2685, 2493, 2217, 1657, 1613, 1590, 1552, 1511, 1470, 1402, 1331, 1252, 1215, 1177, 1161, 1137, 1094, 1066, 1017, 988, 846, 769, 738, 696, 622, 584, 559,537, 460 cm^{-1}. **HRMS** $C_{35}H_{42}FN_3O_6S$; calculated $(M+H)^+$ = 652.2851; average observed $(M+H)^+$ = 652.2852.

Sultam **5** (100 mg, 0.153 mmol) was suspended in DCM and pH 7 phosphate buffer (4:1, 7.65 mL, 0.02 M) and cooled to 0°C. DDQ (52.2 mg, 0.230 mmol) was added in one portion and the solution was allowed to slowly warm to room temperature and stirred overnight (16 h). The reaction was quenched by addition of saturated $NaHCO_3$ solution, extracted with DCM (3x), dried over sodium sulfate, filtered, and concentrated. The crude product was purified by flash chromatography (Isco, silica 4 g, 0-20% MeOH in DCM, 32 min) to give the final product, **BRD2716**, as a light yellow solid (61 mg, 75%). $[\alpha]_D^{20}$ = -23.8° (c 1.0, $CHCl_3$). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.83 (dd, *J* = 7.6, 4.4 Hz, 1H), 7.58 – 7.43 (m, 2H), 7.39 – 7.29 (m, 1H), 7.19 (d, *J* = 19.6 Hz, 1H), 7.07 (t, *J* = 7.9 Hz, 2H), 4.56 (d, *J* = 63.6 Hz, 1H), 4.29 – 3.85 (m, 4H), 3.72 (dt, *J* = 21.2, 9.7 Hz, 1H), 3.60 (d, *J* = 11.3 Hz, 2H), 3.46 (q, *J* = 14.2 Hz, 2H), 3.33 (d, *J* = 14.6 Hz, 1H), 3.22 – 3.03 (m, 4H), 2.70 (s, 3H), 2.50 (s, 3H), 2.26 (s, 1H), 1.09 (dd, *J* = 34.2, 6.6 Hz, 3H), 0.99 (dd, *J* = 14.6, 7.0 Hz, 3H). **^{13}C NMR** (100 MHz, Methanol-d_4) Reported as a mixture of rotamers. δ 167.16, 164.28, 161.80, 155.86, 154.07, 137.62, 137.03, 133.66, 133.64, 133.57, 133.55, 128.92, 128.27, 127.84, 127.67, 127.16, 127.01, 126.10, 125.23, 118.46, 118.42, 115.62, 115.61, 115.40, 115.38, 91.35, 90.96, 86.48, 84.57, 83.53, 63.88, 63.72, 58.47, 57.51, 56.75,

45.48, 44.16, 43.43, 35.49, 35.04, 34.99, 32.57, 15.54, 15.03, 13.33, 12.08. **IR** (PTFE) ν_{max} = 3334, 2966, 2935, 2881, 1657, 1590, 1551, 1509, 1471, 1402, 1331, 1277, 1214, 1172, 1156, 1140, 1096, 1063, 1044, 1017, 983, 841, 726, 621, 599, 582, 555, 533 cm^{-1}. **HRMS** $C_{27}H_{34}FN_3O_5S$, calculated $(M+H)^+$ = 532.2276; average observed $(M+H)^+$ = 532.2270.

Scheme 3. Synthesis of BRD34009.



Procedure: Lactam **6** was prepared according to literature procedure (4). 2,6-Lutidine (0.127 mL, 1.10 mmol) and TBSOTf (0.188 mL, 0.820 mmol) were added to a solution of lactam **6** (162 mg, 0.273 mmol) in DCM (5.5 mL, 0.05 M) at room temperature. The mixture was stirred until complete consumption of starting material was observed by LCMS (2 h). The reaction was quenched with saturated $NaHCO_3$ solution and extracted with DCM (2x). The combined organic extracts were dried over sodium sulfate, filtered, and concentrated to give the crude silyl carbamate. The resulting oil was dissolved in THF (5.5 mL, 0.05 M), HF/pyridine (70% w/w, 0.35 mL, 0.273 mmol) was added, and the resulting mixture stirred for 1 h at room temperature. The reaction was quenched by dropwise addition of saturated $NaHCO_3$ solution and extracted with EtOAc. The combined organic extracts were dried over sodium sulfate, filtered, and concentrated to provide the secondary amine, which was used without further purification.

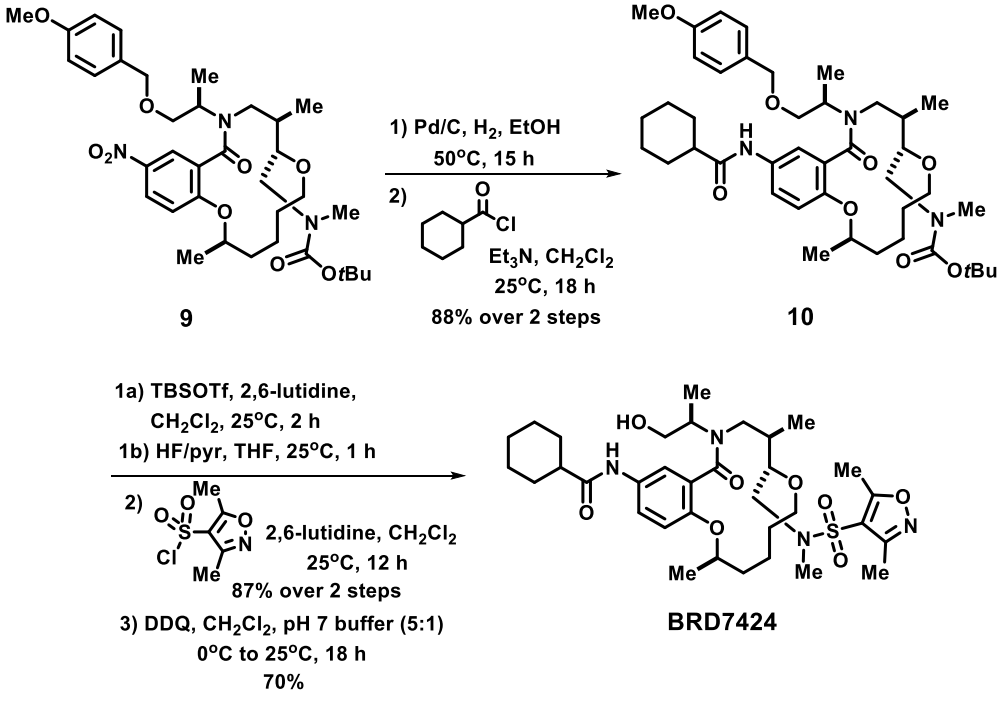
Crude amine (135 mg, 0.274 mmol) was suspended in DMF:AcOH (98:2, 9.2 mL, 0.03 M) and biphenyl-4-carbaldehyde (250 mg, 1.371 mmol) was added. The resulting mixture was stirred at room temperature for 1 h and then sodium triacetoxyborohydride (291 mg, 1.371 mmol) was added and the solution stirred at room temperature for 12 h. The reaction was diluted with EtOAc, quenched by dropwise addition of saturated $NaHCO_3$ solution, and extracted with EtOAc. The combined organic extracts were dried over sodium sulfate, filtered, and concentrated to provide the crude product which was purified by flash chromatography (Isco, silica 4 g, 0-10% MeOH in DCM, 30 min) to give lactam **7** as an off-white solid (130 mg, 72% over two steps).

Lactam **7** (130 mg, 0.197 mmol) was suspended in DMF (4.0 mL, 0.05 M) and Et_3N (1.4mL, 9.87 mmol). N,N-dimethylprop-2-yn-1-amine (0.106 mL, 0.987 mmol) was added at room temperature, followed by $Pd(PPh_3)Cl_2$

(34.6 mg, 0.049 mmol) and finally CuI (37.6 mg, 0.197 mmol). The reaction vessel was purged and backfilled with N_2 (2x) and was then heated to 60°C for 36 h. The reaction mixture was cooled to room temperature, quenched with water, extracted with ethyl acetate, dried over sodium sulfate, and condensed *in vacuo*. The resulting product was purified by flash chromatography (Isco, silica 12 g, 0-15% MeOH in DCM, 36 min) to give dimethylamine **8** as a yellow oil (104 mg, 80%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.37 (s, 1H), 7.85 (s, 1H), 7.48 (dd, *J* = 11.6, 8.1 Hz, 4H), 7.41 (s, 2H), 7.34 (t, *J* = 7.2 Hz, 2H), 7.24 (t, *J* = 6.7 Hz, 1H), 7.21 – 7.08 (m, 3H), 6.76 (d, *J* = 8.1 Hz, 2H), 4.37 (q, *J* = 11.3 Hz, 3H), 4.07 (s, 1H), 3.77 (s, 1H), 3.69 (s, 4H), 3.55 (s, 1H), 3.40 (s, 2H), 3.27 (s, 1H), 3.12 – 2.94 (m, 2H), 2.84 – 2.66 (m, 2H), 2.42 (s, 2H), 2.28 (s, 6H), 2.22 (s, 3H), 2.15 (s, 1H), 1.24 (d, *J* = 6.5 Hz, 3H), 0.61 (s, 3H).**^{13}C NMR** (100 MHz, $CDCl_3$) δ 166.80, 159.22, 153.30, 141.64, 140.92, 130.15, 129.85, 129.33, 128.74, 127.18, 127.02, 126.97, 113.78, 81.15, 73.51, 72.76, 71.92, 69.68, 55.25, 52.19, 48.51, 48.36, 44.19, 44.09, 35.75, 16.40, 14.76. **IR** (PTFE) ν_{max} = 3386, 3028, 2938, 2858, 2776, 1634, 1593, 1554, 1513, 1452, 1386, 1357, 1303, 1272, 1248, 1212, 1173, 1157, 1085, 1035, 1011, 971, 926, 820, 762, 737, 699 cm^{-1}. **HRMS** $C_{41}H_{48}N_4O_4$; calculated $(M+H)^+$= 661.3748; average observed $(M+H)^+$= 661.3754.

Dimethylamine **8** (21 mg, 0.032 mmol) was suspended in DCM (1.6 mL, 0.02 M) and cooled to 0°C. Triethylsilane (10.2 µL, 0.064 mmol) was added, followed by boron trifluoride diethyletherate (32.2 µL, 0.254 mmol) and the reaction was monitored by LCMS and stirred until completion (3 h). Methanol was added dropwise (1.0 mL) and the mixture was condensed *in vacuo* to give a yellow oil, which was purified by flash chromatography (Isco, silica, 4 g, 0-20% MeOH in DCM, 36 min) to give the final product. **BRD34009**, as a white solid (15.0 mg, 87%). $[\alpha]_D^{20}$ = +71.9° (c 1.0, MeOH). **^{1}H NMR** (400 MHz, Methanol-d_4) δ 8.65 (s, 1H), 8.08 (s, 1H), 7.74 (q, *J* = 7.3 Hz, 4H), 7.66 (d, *J* = 7.2 Hz, 2H), 7.47 (t, *J* = 7.2 Hz, 2H), 7.38 (t, *J* = 6.1 Hz, 1H), 5.06 (s, 1H), 4.56 (t, *J* = 9.0 Hz, 1H), 4.39 – 4.16 (m, 2H), 3.92 (s, 3H), 3.79 – 3.49 (m, 4H), 3.48 – 3.34 (m, 1H), 3.25 (d, *J* = 16.0 Hz, 1H), 2.99 (s, 2H), 2.68 (s, 6H), 2.40 – 2.28 (m, 1H), 1.33 (d, *J* = 6.4 Hz, 3H), 0.93 (d, *J* = 6.5 Hz, 3H).**^{13}C NMR** (100 MHz, Methanol-d_4) δ 166.99, 160.06, 153.47, 142.74, 142.11, 139.90, 131.54, 128.62, 127.56, 127.34, 126.65, 124.48, 117.56, 84.74, 82.13, 62.98, 58.33, 56.34, 52.20, 42.33, 40.05, 36.05, 14.40, 13.10. **IR** (PTFE) ν_{max} = 3350, 3031, 2962, 2923, 2852, 1623, 1560, 1453, 1371, 1206, 1150, 1061, 960, 934, 815, 766, 739, 701, 554, 513 cm^{-1}. **HRMS** $C_{33}H_{40}N_4O_3$; calculated $(M+H)^+$ = 541.3173; average observed $(M+H)^+$ = 541.3185.

Scheme 4. Synthesis of BRD7424.



Procedure: Macrocycle **9** was prepared according to literature procedure (5). Macrocycle **9** (160 mg, 0.244 mmol) was suspended in EtOH (12.2 mL, 0.02 M) in a 10-20 mL microwave vial and activated 10% Pd/C (2.6 mg, 0.024 mmol) was added at room temperature. The vial was sealed and then evacuated and refilled with N_2 (2x). The vial was evacuated a third time, a hydrogen balloon was added, and then the stirring mixture was heated to 50°C for 15 h. The reaction mixture was cooled, filtered through celite, and the celite was liberally washed with excess EtOAc. The filtrate was condensed *in vacuo* to give a pale purple foaming solid that was carried on to the next step without further purification.

Crude aniline (100 mg, 0.159 mmol) was suspended in DCM (8.0 mL, 0.02 M) and Et_3N (0.067 mL, 0.478 mmol) and cyclohexanecarbonyl chloride (0.026 mL, 0.191 mmol) were added at room temperature, and the resulting mixture was stirred for 18 h. The reaction was quenched by dropwise addition of saturated $NaHCO_3$ solution and extracted with DCM (2x). The combined organic extracts were dried over sodium sulfate, filtered, and concentrated to provide the crude product which was purified by flash chromatography (Isco, silica 4 g, 0-30% EtOAc in Hexanes, 30 min) to give amide **10** as a white solid (104 mg, 88% over two steps). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.70 (s, 1H), 7.27 (d, *J* = 8.4 Hz, 2H), 7.20 (d, *J* = 5.2 Hz, 1H), 7.01 (s, 1H), 6.87 (d, *J* = 8.1 Hz, 2H), 6.78 (d, *J* = 8.4 Hz, 1H), 4.67 (s, 1H), 4.56 – 4.41 (m, 2H), 4.41 – 4.26 (m, 1H), 3.96 (d, *J* = 14.2 Hz, 1H), 3.80 (s, 3H), 3.70 – 3.53 (m, 2H), 3.53 – 3.34 (m, 2H), 3.32 – 3.17 (m, 1H), 3.05 – 2.79 (m, 3H), 2.62 (d, *J* = 20.7 Hz, 2H), 2.21 (s, 1H), 2.08 (d, *J* = 22.9 Hz, 1H), 1.95 – 1.63 (m, 9H), 1.52 (s, 3H), 1.43 (d, *J* = 20.7 Hz, 9H), 1.36 – 1.30 (m, 3H), 1.29 – 1.23 (m, 3H), 1.22 (s, 2H), 1.09 (s, 3H), 0.89 (s, 3H). **IR** (PTFE) ν_{max} = 3297, 2928, 2855, 2361, 1691, 1612, 1541, 1513, 1492, 1452, 1393, 1367, 1248, 1205, 1151, 1098, 1036, 928, 882, 819 cm^{-1}. **HRMS** $C_{42}H_{63}N_3O_8$; calculated $(M+H)^+$ = 738.4688; average observed $(M+H)^+$ = 738.4704.

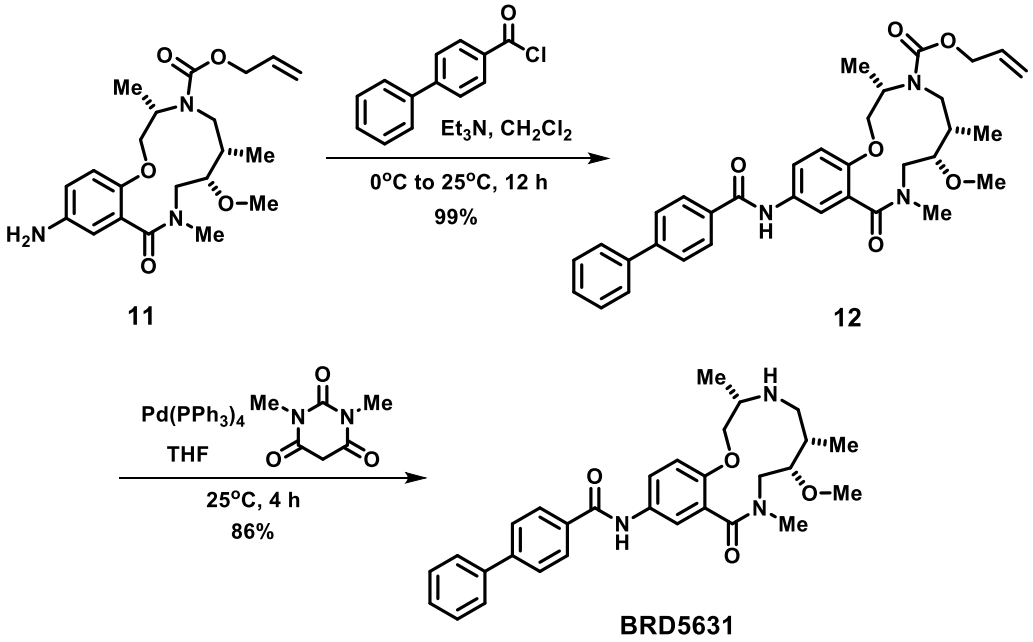
2,6-lutidine (0.51 mL, 0.434 mmol) and TBSOTf (0.075 mL, 0.325 mmol) were added to a solution of amide **10** (80 mg, 0.108 mmol) in DCM (2.15 mL, 0.05 M) at room temperature. The mixture was stirred until complete consumption of starting material was observed by LCMS (2 h). The reaction was quenched with saturated $NaHCO_3$ solution and extracted with DCM (2x). The combined organic extracts were dried over sodium sulfate, filtered, and concentrated to give the silyl carbamate. The crude oil was dissolved in THF (2.15 mL, 0.05 M), HF/pyridine (70% w/w, 0.014 mL, 0.108 mmol) was added, and the resulting mixture stirred for 1 h at room temperature. The reaction was quenched by dropwise addition of saturated $NaHCO_3$ solution and extracted with EtOAc. The combined organic extracts were dried over sodium sulfate, filtered, and concentrated to provide the secondary amine, which was used without further purification.

Crude amine (69 mg, 0.108 mmol) was suspended in DCM (5.4 mL, 0.02 M) and 2,6-lutidine (0.025 mL, 0.216 mmol) and 3,5-dimethylisoxazole-4-sulfonyl chloride (106 mg, 0.541 mmol) were added at room temperature, and the resulting mixture was stirred for 12 h. The reaction was quenched by dropwise addition of saturated $NaHCO_3$ solution and extracted with DCM (2x). The combined organic extracts were dried over sodium sulfate, filtered, and concentrated to provide the crude product which was purified by flash chromatography (Isco, silica 4 g, 0-30% EtOAc in Hexanes, 30 min) to give the sulfonamide as a white solid (75 mg, 87%).

The sulfonamide (25 mg, 0.031 mmol) was suspended in DCM and pH 7 phosphate buffer (4:1, 3.1 mL, 0.01 M) and cooled to 0°C. DDQ (10.68 mg, 0.047 mmol) was added in one portion and the solution was allowed to slowly warm to room temperature and stirred overnight (18 h). The reaction was quenched by addition of saturated $NaHCO_3$ solution, extracted with DCM (3x), dried over sodium sulfate, filtered, and concentrated. The crude product was purified by flash chromatography (Isco, silica 4 g, 0-10% MeOH in DCM, 32 min) to give the final product, **BRD7424**, as a white solid (13 mg, 61%). $[\alpha]_D^{20}$ = -7.20° (c 1.0, $CHCl_3$). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.68 (d, J = 7.9 Hz, 1H), 7.46 (d, J = 5.7 Hz, 1H), 7.33 (s, 1H), 6.83 (d, J = 8.6 Hz, 1H), 4.75 (s, 1H), 4.38 (s, 1H), 4.33 – 4.05 (m, 1H), 4.05 – 3.88 (m, 2H), 3.88 – 3.75 (m, 1H), 3.74 – 3.59 (m, 2H), 3.57 – 3.43 (m, 4H), 3.28-3.21 (m, 2H), 3.09-3.00 (m, 1H), 2.97 (s, 3H), 2.90 – 2.75 (m, 1H), 2.66 (s, 3H), 2.59 (s, 1H), 2.48 (s, 1H), 2.42 (s, 3H), 2.35 (s, 1H), 2.28 – 2.13 (m, 1H), 1.95-1.66 (m, 8H), 1.58 – 1.47 (m, 3H), 1.40 (d, J = 5.5 Hz, 3H), 1.36 – 1.23 (m, 4H), 1.09 (d, J = 6.8 Hz, 3H), 1.01 (d, J = 6.4 Hz, 3H), 0.93-0.90 (m, m,

2H).**^{13}C NMR** (100 MHz, $CDCl_3$) δ 174.36, 173.47, 172.95, 157.65, 150.34, 131.04, 122.55, 120.47, 115.17, 113.68, 85.29, 81.80, 75.60, 70.65, 63.62, 57.52, 49.34, 46.17, 44.27, 36.57, 33.72, 32.56, 29.65, 29.60, 28.30, 26.66, 25.68, 25.63, 19.74, 18.64, 18.18, 17.65, 15.26, 14.19, 12.93, 11.40, 11.28. **IR** (PTFE) ν_{max} = 3270, 2927, 2855, 2361, 2339, 1594, 1540, 1494, 1450, 1407, 1373, 1340, 1206, 1152, 754, 629, 512 cm^{-1}. **HRMS** $C_{34}H_{52}N_4O_8S$, calculated $(M+H)^+$ = 677.3579; average observed $(M+H)^+$ = 677.3587.

Scheme 5. Synthesis of BRD5631.

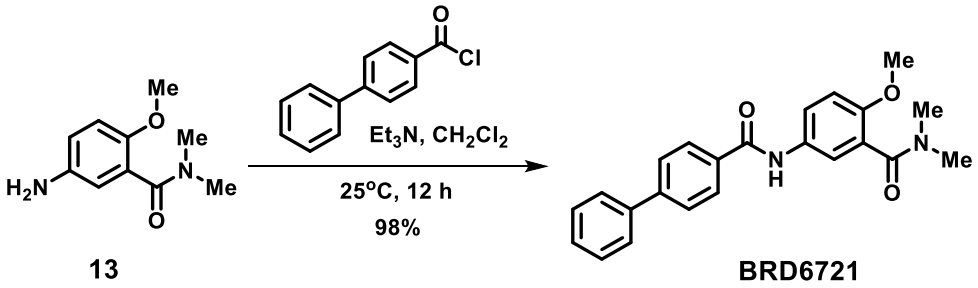


Procedure: Macrocycle **11** was prepared according to literature procedure (6). Macrocycle **11** (260 mg, 0.641 mmol) was suspended in DCM (32 mL, 0.02 M) and cooled to 0°C. Et_3N (0.27 mL, 1.924 mmol) was added, followed by biphenyl-4-carbonyl chloride (278 mg, 1.282 mmol). After 0.5 h, the reaction was warmed to room temperature and stirred for an additional 1 h. The reaction was quenched by addition of saturated $NaHCO_3$, extracted with DCM (2x), condensed *in vacuo*, and purified by flash chromatography (Isco, silica 24 g, 0-100% EtOAc in Hexanes, 32 min) to give biphenyl amide **12** as a white solid (378 mg, 99%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 9.38 (d, *J* = 21.3 Hz, 1H), 8.05 (dd, *J* = 35.5, 6.9 Hz, 2H), 7.74 (s, 1H), 7.63 (dd, *J* = 17.8, 7.5 Hz, 5H), 7.45 (d, *J* = 7.2 Hz, 2H), 7.39 (d, *J* = 6.5 Hz, 1H), 7.22 (s, 1H), 6.75 (dd, *J* = 66.3, 7.5 Hz, 1H), 5.92 (s, 1H), 5.30 (t, *J* = 19.6 Hz, 1H), 5.19 (d, *J* = 10.1 Hz, 1H), 4.53 (d, *J* = 21.0 Hz, 3H), 4.13 – 3.98 (m, 1H), 3.88 – 3.62 (m, 2H), 3.42-3.32 (m, 6H), 3.20 (s, 3H), 3.05 (d, *J* = 31.3 Hz, 1H), 1.41 – 1.24 (m, 1H), 0.99 (d, *J* = 40.5 Hz, 6H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 170.23, 169.91, 165.61, 156.90, 151.16, 150.50, 144.42, 140.03, 139.95, 133.37, 133.28, 133.13, 133.07, 132.94, 131.73, 128.94, 128.12, 128.07, 128.01, 127.89, 127.18, 127.15, 127.11, 125.48, 123.72, 120.24, 119.65, 117.09, 117.05, 113.16, 110.93, 86.74, 70.67, 66.12, 65.96, 65.58, 60.39, 58.48, 51.45, 49.95, 38.63, 35.64, 34.01, 33.27, 21.05, 18.86, 18.01, 14.20, 14.05. **IR** (PTFE) ν_{max} = 3303, 2974, 2931, 1692, 1613, 1542, 1498, 1462, 1411, 1328, 1220, 1154, 1096, 1071, 1042, 991, 897, 855, 817, 772, 746, 698, 636, 553, 512 cm^{-1}. **HRMS** $C_{34}H_{39}N_3O_6$; calculated $(M+H)^+$ = 586.2912; average observed $(M+H)^+$ = 586.2913.

Biphenyl amide **12** (325 mg, 0.555 mmol) was suspended in THF (13.9 mL, 0.04 M) and 1,3-dimethylbarbituric acid (173 mg, 1.110 mmol) was added, followed by $Pd(PPh_3)_4$ (128 mg, 0.111 mmol). The resulting solution was stirred at room temperature until the reaction reached completion (4 h) and then the mixture was condensed *in vacuo* and purified by flash chromatography (Isco, silica 12 g, 0-15% MeOH in DCM, 36 min) to give the final product, **BRD5631**, as a pale pink solid (278 mg, 86%). $[\alpha]_D^{20}$ = -36.9° (c 1.0, $CHCl_3$). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.62 (s, 1H), 8.18 – 8.07 (m, 0H), 8.00 (d, *J* = 7.6 Hz, 2H), 7.71-7.61 (m, 5H), 7.48 – 7.45 (m, 2H), 7.41 – 7.36 (m, 2H), 6.97 (dd, *J* = 44.1, 8.5 Hz, 1H), 6.79 (d, *J* = 8.9 Hz, 1H), 4.61 (dd, *J* = 54.3, 14.2

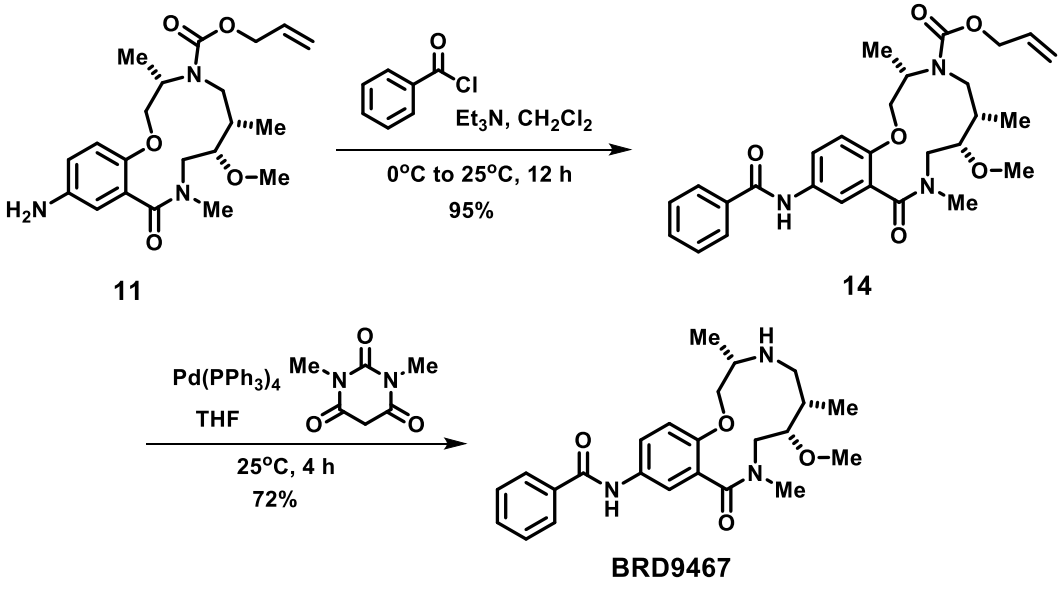
Hz, 1H), 4.03 – 3.90 (m, 1H), 3.75 (d, *J* = 9.1 Hz, 1H), 3.56 – 3.46 (m, 1H), 3.39 (d, *J* = 21.4 Hz, 2H), 3.33 (s, 3H), 3.25 – 3.16 (m, 1H), 3.09 (s, 3H), 2.97-2.94 (m, 2H), 2.76 – 2.54 (m, 2H), 2.45 – 2.33 (m, 1H), 2.24 (s, 1H), 1.02 (s, 6H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 170.38, 165.11, 149.71, 144.27, 140.04, 139.98, 133.30, 131.55, 128.94, 128.00, 127.90, 127.15, 127.04, 127.00, 125.22, 123.27, 120.91, 110.91, 82.54, 74.23, 59.09, 58.31, 53.43, 49.19, 47.58, 45.02, 39.37, 37.06, 31.67, 28.77, 18.78, 16.31. **IR** (PTFE) ν_{max} = 3301, 3131, 3056, 2962, 2931, 2875, 1614, 1542, 1489, 1458, 1406, 1309, 1261, 1196, 1166, 1093, 1072, 1021, 1008, 897, 855, 814, 781, 746, 699, 664, 622, 584, 551 cm^{-1}. **HRMS** $C_{30}H_{35}N_3O_4$; calculated $(M+H)^+$ = 502.2700; average observed $(M+H)^+$ = 502.2699.

Scheme 6. Synthesis of BRD6721.



Procedure: 5-amino-2-methoxy-N,N-dimethylbenzamide (**13**) (100 mg, 0.515 mmol) was suspended in DCM (5.2 mL, 0.1 M) and Et_3N (0.144 mL, 1.030 mmol) was added, followed by biphenyl-4-carbonyl chloride (134 mg, 0.618 mmol) at room temperature. The reaction was stirred for 16 h at room temperature, quenched with water, and extracted with DCM (2x). The combined organic extracts were dried over sodium sulfate, filtered, concentrated, and purified by flash chromatography (Isco, silica 12 g, 0-10% MeOH in DCM, 32 min) to give the final product, **BRD6721**, as a pale pink solid (188 mg, 98%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.98 (s, 1H), 8.05 (d, *J* = 7.8 Hz, 2H), 7.80 (d, *J* = 8.5 Hz, 1H), 7.66 (dd, *J* = 16.6, 7.7 Hz, 4H), 7.47 (t, *J* = 7.2 Hz, 2H), 7.40 (d, *J* = 6.8 Hz, 1H), 7.31 (s, 1H), 6.82 (d, *J* = 8.9 Hz, 1H), 3.71 (s, 3H), 3.10 (s, 3H), 2.88 (s, 3H). **^{13}C NMR** (75 MHz, $CDCl_3$) δ 169.37, 165.42, 151.91, 144.32, 140.10, 133.33, 131.68, 128.88, 128.84, 127.98, 127.94, 127.18, 127.15, 127.08, 125.04, 123.63, 121.11, 111.22, 55.78. **IR** (PTFE) ν_{max} = 3318, 2928, 1664, 1621, 1540, 1504, 1462, 1443, 1411, 1392, 1328, 1317, 1289, 1260, 1230, 1186, 1151, 1108, 1079, 1029, 1005, 923, 904, 884, 868, 854, 824, 781, 766, 749, 702, 655, 641, 613, 568, 547, 511, 492, 447 cm^{-1}. **HRMS** $C_{23}H_{22}N_2O_3$; calculated $(M+H)^+$ = 375.1703; average observed $(M+H)^+$ = 375.1703.

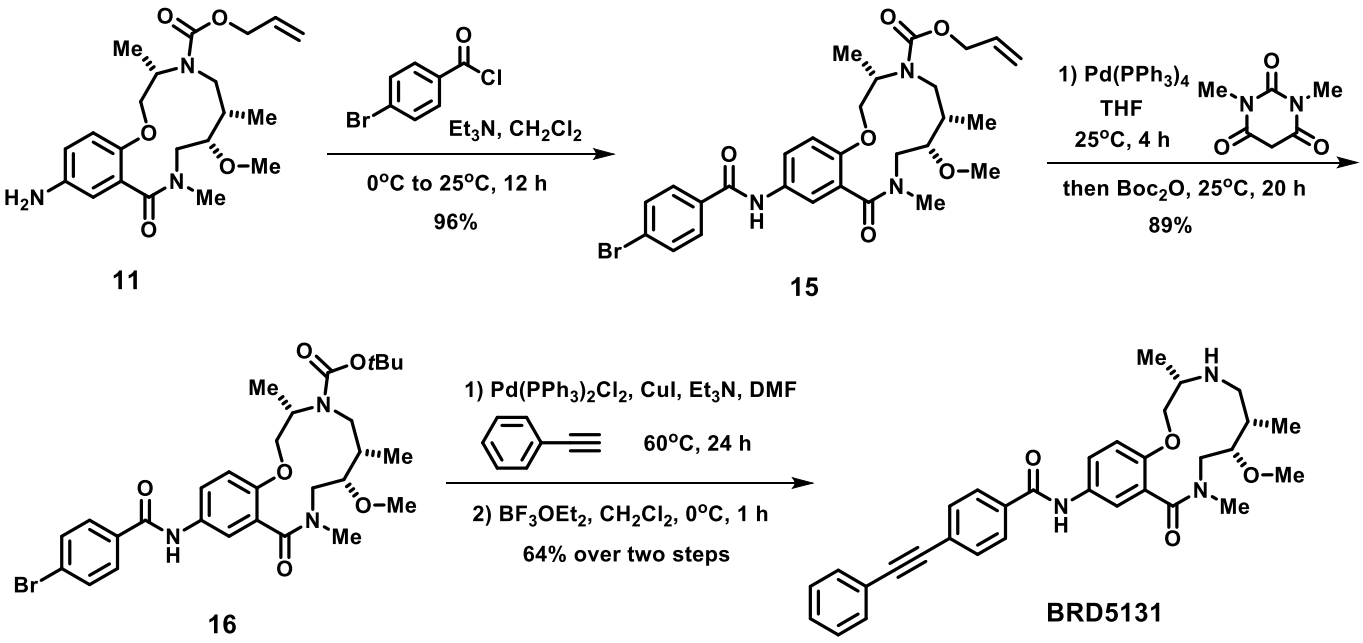
Scheme 7. Synthesis of BRD9467.



Procedure: Macrocycle **11** (25 mg, 0.062 mmol) was suspended in DCM (3.1 mL, 0.02 M) and cooled to 0°C. Et_3N (0.013 mL, 0.092 mmol) was added, followed by biphenyl-4-carbonyl chloride (0.009 mL, 0.074 mmol). After 0.5 h, the reaction was warmed to room temperature and stirred for 16 h. The reaction was quenched by addition of saturated $NaHCO_3$, extracted with DCM (2x), concentrated, and purified by flash chromatography (Isco, silica 4 g, 0-100% EtOAc in Hexanes, 32 min) to give phenyl amide **16** as a white solid (30 mg, 95%).

Phenyl amide **14** (30 mg, 0.059 mmol) was suspended in THF (3.0 mL, 0.02 M) and 1,3-dimethylbarbituric acid (18.4 mg, 0.118 mmol) was added, followed by $Pd(PPh_3)_4$ (13.6 mg, 0.012 mmol). The resulting solution was stirred at room temperature until the reaction reached completion (4 h) and then the mixture was condensed *in vacuo* and purified by flash chromatography (Isco, silica 4 g, 0-15% MeOH in DCM, 36 min) to give the final product, **BRD9467**, as a pale pink solid (18 mg, 72%). **^{1}H NMR** (400 MHz, $CDCl_3$) Reported as a mixture of rotamers. δ 8.55 (s, 1H), 7.96 (dd, *J* = 35.2, 6.2 Hz, 2H), 7.76 – 7.10 (m, 5H), 7.10 – 6.69 (m, 1H), 4.84 – 4.48 (m, 1H), 4.00 – 3.79 (m, 1H), 3.78 – 3.61 (m, 1H), 3.61 – 3.46 (m, 1H), 3.40 (d, *J* = 18.6 Hz, 1H), 3.34 (s, 2H), 3.19 (dd, *J* = 14.5, 7.8 Hz, 1H), 3.09 (d, *J* = 12.4 Hz, 2H), 2.96 (d, *J* = 7.9 Hz, 1H), 2.65 (dd, *J* = 24.3, 14.5 Hz, 1H), 2.50 – 2.31 (m, 1H), 2.26 (d, *J* = 37.1 Hz, 1H), 2.02 (d, *J* = 17.7 Hz, 1H), 1.25 (s, 2H), 1.18 (s, 1H), 1.03 (s, 3H), 0.83 (s, 1H). **IR** (PTFE) ν_{max} = 3283, 3060, 2958, 2925, 2873, 2855, 1663, 1619, 1540, 1496, 1460, 1401, 1308, 1258, 1213, 1151, 1095, 1073, 1027, 730, 709 cm^{-1}. **HRMS** $C_{24}H_{31}N_3O_4$; calculated $(M+H)^+$ = 426.2387; average observed $(M+H)^+$ = 426.2390.

Scheme 8. Synthesis of BRD5131.



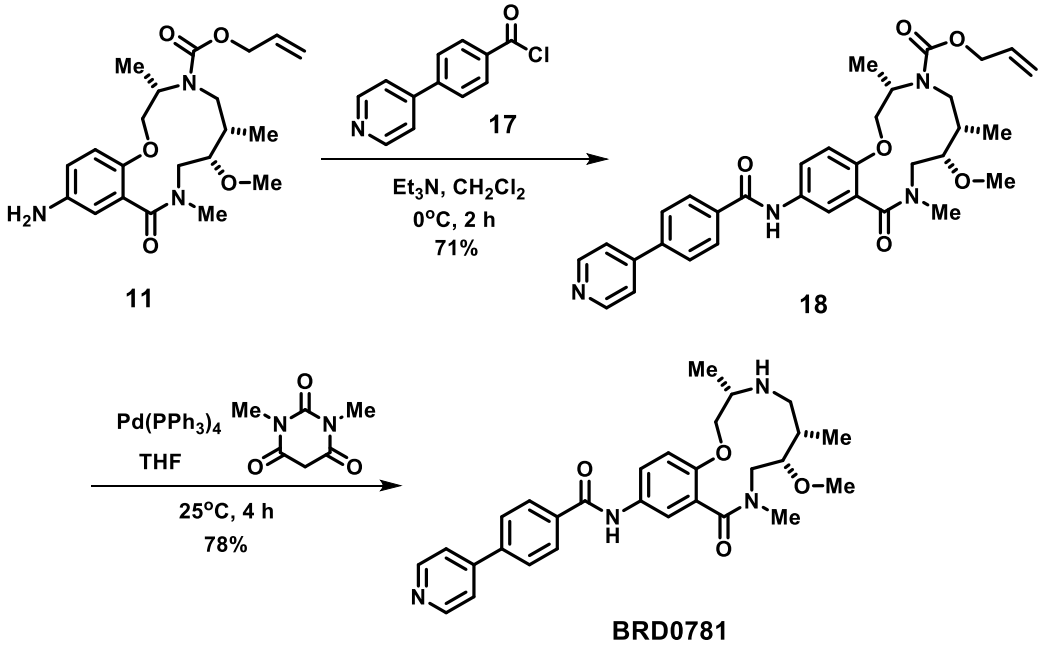
Procedure: Macrocycle **11** (50 mg, 0.123 mmol) was suspended in DCM (6.17 mL, 0.02 M) and cooled to 0°C. Et_3N (0.026 mL, 0.185 mmol) was added, followed by 4-bromobenzoyl chloride (32.5 mg, 0.148 mmol). After 0.5 h, the reaction was warmed to room temperature and stirred for 16 h. The reaction was quenched by addition of water, extracted with DCM (2x), and condensed *in vacuo* to give 4-bromobenzoyl amide **15** as a white solid (70 mg, 96%) which was carried on to the next step without further purification.

4-bromobenzoyl amide **15** was suspended in THF (6.0 mL, 0.02 M) and 1,3-dimethylbarbituric acid (37.1 mg, 0.238 mmol) was added, followed by $Pd(PPh_3)_4$ (27.5 mg, 0.024 mmol). The resulting solution was stirred at room temperature until the reaction reached completion (4 h), and then di-*t*-butyl dicarbonate (78 mg, 0.357 mmol) was added and the resulting solution stirred 20 h at room temperature. The resulting mixture was condensed *in vacuo* and purified by flash chromatography (Isco, silica 12 g, 0-25% MeOH in DCM, 45 min) to give carbamate **16** as an orange solid (64 mg, 89%).

Carbamate **16** (30 mg, 0.05 mmol) was suspended in DMF (0.827 mL, 0.06 M) and Et_3N (0.173 mL, 1.241 mmol). Phenylacetylene (27.2 µL, 0.248 mmol) was added at room temperature, followed by $Pd(PPh_3)Cl_2$ (8.71 mg, 0.012 mmol) and finally CuI (14.18 mg, 0.074 mmol). The reaction vessel was purged and backfilled with N_2 (2x) and was then heated to 60°C for 18 h. The reaction mixture was cooled to room temperature, quenched with water, extracted with EtOAc, dried over sodium sulfate, and condensed *in vacuo*. The resulting product was purified by flash chromatography (Isco, silica 4 g, 0-100% EtOAc in Hexanes, 42 min) to give diphenyl alkyne as a light brown oil (22 mg, 71%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.08 – 7.76 (m, 2H), 7.73 – 7.41 (m, 4H), 7.37 (s, 2H), 7.04 – 6.62 (m, 4H), 4.74 – 4.38 (m, 1H), 4.08 (s, 1H), 3.87 (s, 1H), 3.47 – 3.24 (m, 4H), 3.20 (s, 3H), 3.07 – 2.84 (m, 2H), 2.19 – 1.94 (m, 1H), 1.47 (s, 9H), 1.34 – 1.21 (m, 2H), 1.22 – 1.07 (m, 3H), 0.98 (s, 3H). **IR** (PTFE) ν_{max} = 3287, 3059, 2972, 2929, 1679, 1611, 1543, 1502, 1461, 1410, 1367, 1332, 1211, 1153, 1097, 1071, 1041, 992, 906, 854, 812, 758, 692, 637, 540, 513 cm^{-1}. **HRMS** $C_{37}H_{43}N_3O_6$; calculated $(M+Na)^+$ = 648.3044; average observed $(M+Na)^+$ = 648.3074.

The diphenyl alkyne intermediate was suspended in DCM (1.76 mL, 0.01 M) and cooled to 0°C. Boron trifluoride diethyletherate (17.82 µl, 0.141 mmol) was added dropwise and the reaction was stirred until complete by LCMS (1 h). The reaction was quenched by the addition of MeOH (200 µL), the solvent evaporated, and the crude product purified by flash chromatography (Isco, silica 4 g, 0-20% MeOH in DCM, 32 min) to give the final product, **BRD5131**, as an off-white solid (8.2 mg, 89%, 64% over two steps). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.97 (s, 1H), 8.44 – 8.16 (m, 1H), 8.02 (d, *J* = 7.9 Hz, 2H), 7.80 – 7.39 (m, 5H), 7.41 – 7.30 (m, 3H), 6.96 (d, *J* = 8.5 Hz, 1H), 4.66 – 4.34 (m, 1H), 3.91 (s, 1H), 3.69-3.61 (m, 1H), 3.46-3.31 (m, 3H), 3.19 (d, *J* = 14.4 Hz, 1H), 2.94 (s, 3H), 2.78 (s, 1H), 2.56 (s, 1H), 1.44 (s, 3H), 1.25-1.06 (m, 5H), 0.91 – 0.82 (m, 1H). **IR** (PTFE) ν_{max} = 3364, 3201, 3062, 2926, 2853, 1608, 1541, 1493, 1467, 1419, 1397, 1304, 1285, 1254, 1208, 1153, 1076, 813, 760, 691, 513 cm^{-1}. **HRMS** $C_{32}H_{35}N_3O_4$; calculated $(M+H)^+$ = 526.2700; average observed $(M+H)^+$ = 526.2701.

Scheme 9. Synthesis of BRD0781.

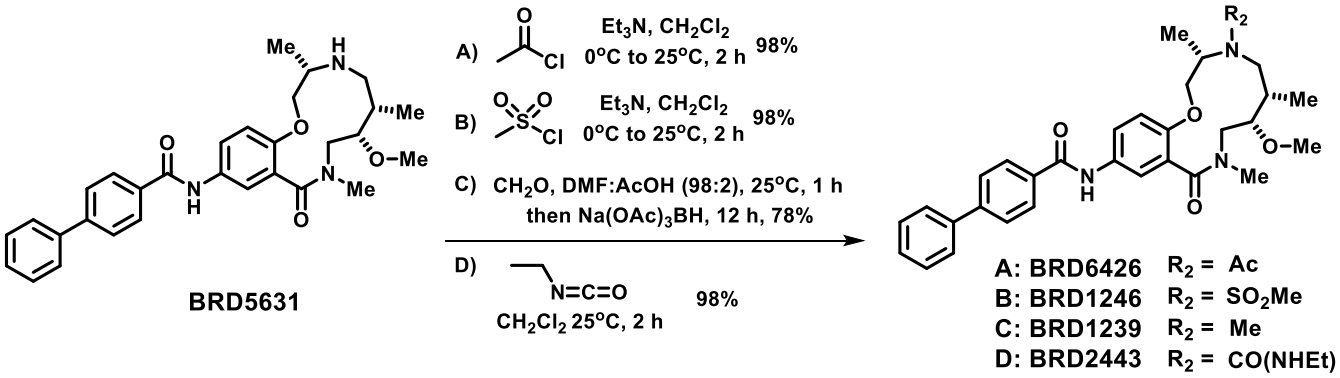


Procedure: 4-(pyridin-4-yl)benzoic acid (34.4 mg, 0.173 mmol) was suspended in DCM (2.16 mL, 0.04 M) and oxalylchloride (15.55 µL, 0.181 mmol) was added, followed by dropwise addition of catalytic N,N-dimethylacetamide (1.593 µL, 0.017 mmol). The reaction was stirred until it no longer bubbled (1 h), then in a separate flask, macrocycle **11** (35.0 mg, 0.086 mmol) was suspended in DCM (2.16 mL, 0.02 M) and Et_3N (48.1 µl, 0.345 mmol) was added. This mixture was cooled to 0°C and the solution of acid chloride **17** was added dropwise. The reaction was stirred at 0°C for 2 h and was then quenched by addition of water, extracted

with DCM, washed with 1.0 M NaOH, dried over sodium sulfate, and condensed *in vacuo*. The crude product, 4-(pyridin-4-yl)benzoyl amide **18**, was carried on to the next step without further purification (36 mg, 71%).

4-(pyridin-4-yl)benzoyl amide **18** (33 mg, 0.056 mmol) was suspended in THF (2.8 mL, 0.02 M) and 1,3-dimethylbarbituric acid (17.6 mg, 0.112 mmol) was added, followed by $Pd(PPh_3)_4$ (9.8 mg, 0.0084 mmol). The resulting solution was stirred at room temperature until the reaction reached completion (6 h) and then the mixture was condensed *in vacuo* and purified by flash chromatography (Isco, silica 4 g, 0-25% MeOH in DCM, 32 min) to give the final product, **BRD0781**, as a light orange solid (22 mg, 78%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.63 (s, 1H), 8.04 (d, *J* = 7.0 Hz, 2H), 7.90 – 7.30 (m, 7H), 7.09 – 6.66 (m, 1H), 4.07 – 3.82 (m, 1H), 3.82 – 3.64 (m, 1H), 3.56 – 3.46 (m, 1H), 3.41 (d, *J* = 9.9 Hz, 1H), 3.34 (s, 3H), 3.29 – 3.17 (m, 1H), 3.10 (s, 3H), 2.98 (d, *J* = 16.1 Hz, 2H), 2.72 – 2.59 (m, 1H), 2.49 – 2.34 (m, 1H), 2.28 – 2.13 (m, 1H), 1.25 (s, 3H), 1.01 (s, 3H), 0.90 – 0.81 (m, 1H). **IR** (PTFE) ν_{max} = 3299, 3060, 2958, 2926, 2874, 1662, 1600, 1546, 1493, 1458, 1405, 1310, 1258, 1211, 1152, 1093, 1071, 1017, 822, 763, 512 cm^{-1}. **HRMS** $C_{29}H_{34}N_4O_4$; calculated $(M+H)^+$ = 503.2653; average observed $(M+H)^+$ = 503.2652.

Scheme 10. Synthesis of N-capped Analogues of BRD5631.



Procedure A: BRD5361 (15 mg, 0.030 mmol) was suspended in DCM (1.5 mL, 0.02 M) and cooled to 0°C. Et_3N (12.5 µL, 0.090 mmol) was added, followed by acetyl chloride (3.2 µL, 0.045 mmol). After 0.5 h, the reaction was warmed to room temperature and stirred for 16 h. The reaction was quenched by addition of water, extracted with DCM (2x), condensed *in vacuo*, and purified by flash chromatography (Isco, silica 4 g, 0-10% MeOH in DCM, 32 min) to give acetamide **BRD6426** as a white solid (16 mg, 98%). **^{1}H NMR** (400 MHz, $CDCl_3$) Reported as a mixture of rotamers. δ 9.10 (s, 0.5H), 8.42 (d, *J* = 13.3 Hz, 0.5H), 8.02 (dd, *J* = 34.3, 7.9 Hz, 2H), 7.81 – 7.57 (m, 5H), 7.53 – 7.30 (m, 4H), 6.75 (dd, *J* = 36.5, 8.9 Hz, 1H), 5.09 (d, *J* = 6.3 Hz, 0.36H), 4.95 (t, *J* = 9.4 Hz, 0.36H), 4.59 (d, *J* = 14.3 Hz, 0.36H), 4.28 – 4.00 (m, 1H), 3.96 – 3.65 (m, 2H), 3.48 (t, *J* = 12.1 Hz, 2H), 3.43 – 3.28 (m, 3H), 3.20 (d, *J* = 6.2 Hz, 2H), 3.09 (dd, *J* = 24.6, 14.9 Hz, 2H), 2.85 (s, 1H), 2.81 – 2.67 (m, 1H), 2.25 – 2.07 (m, 3H), 1.37 (d, *J* = 6.7 Hz, 2H), 1.25 (s, 3H), 1.16 – 1.03 (m, 2H), 0.99 (d, *J* = 6.9 Hz, 3H), 0.92 – 0.84 (m, 1H). **IR** (PTFE) ν_{max} = 3450, 3287, 3056, 2969, 2926, 2854, 1615, 1543, 1492, 1462, 1424, 1332, 1278, 1248, 1220, 1151, 1096, 1073, 1039, 990, 954, 895, 855, 818, 746, 699, 636, 609, 584, 511 cm^{-1}. **HRMS** $C_{32}H_{37}N_3O_5$; calculated $(M+H)^+$ = 544.2806; average observed $(M+H)^+$ = 544.2816.

Procedure B: BRD5361 (15 mg, 0.030 mmol) was suspended in DCM (1.5 mL, 0.02 M) and cooled to 0°C. Et_3N (20.8 µL, 0.150 mmol) was added, followed by methanesulfonyl chloride (5.8 µL, 0.075 mmol). After 0.5 h, the reaction was warmed to room temperature and stirred for 1 h. The reaction was quenched by addition of water, extracted with DCM (2x), condensed *in vacuo*, and purified by flash chromatography (Isco, silica 4 g, 0-10% MeOH in DCM, 32 min) to give sulfonamide **BRD1246** as a white solid (17 mg, 98%). **^{1}H NMR** (400 MHz, $CDCl_3$) Reported as a mixture of rotamers. δ 8.78 (s, 0.5 H), 8.54 (d, *J* = 91.3 Hz, 0.5 H), 8.01 (dd, *J* = 19.6, 7.8 Hz, 2H), 7.74 – 7.60 (m, 4H), 7.43 (dt, *J* = 29.4, 7.3 Hz, 3H), 6.88 (dd, *J* = 19.5, 8.7 Hz, 0.5 H), 6.73 (s, 0.5 H), 4.63 – 4.44 (m, 1H), 4.37 – 4.02 (m, 2H), 3.97 – 3.76 (m, 1H), 3.47 – 3.32 (m, 5H), 3.21 (s, 3H), 3.16 – 3.00

(m, 2H), 2.94 (d, *J* = 15.4 Hz, 2H), 2.81 (s, 3H), 1.37 (q, *J* = 7.6, 6.9 Hz, 2H), 1.23 (d, *J* = 12.8 Hz, 3H), 1.18 – 1.10 (m, 1H), 1.06 (d, *J* = 6.8 Hz, 3H), 0.94 – 0.76 (m, 1H). **IR** (PTFE) ν_{max} = 3301, 3031, 2929, 2603, 2496, 1614, 1541, 1492, 1462, 1398, 1324, 1247, 1216, 1144, 1096, 1072, 1033, 994, 961, 820, 777, 747, 699 cm^{-1}. **HRMS** $C_{31}H_{37}N_3O_6S$; calculated $(M+H)^+$ = 580.2476; average observed $(M+H)^+$ = 580.2486.

Procedure C: BRD5631 (15 mg, 0.030 mmol) was suspended in DMF:AcOH (98:2, 1.5 mL, 0.02 M); formaldehyde (11.2 µL, 0.150 mmol) was added and the reaction was stirred for 30 min at room temperature. Sodium triacetoxyborohydride (31.7 mg, 0.150 mmol) was added and the reaction was stirred for an additional 12 h. The reaction was diluted with EtOAc, quenched by dropwise addition of saturated $NaHCO_3$ solution, and extracted with EtOAc (3x). The combined organic extracts were dried over sodium sulfate, filtered, and concentrated to provide the crude product which was purified by flash chromatography (Isco, silica 4 g, 0-15% MeOH in DCM, 26 min) to give **BRD1239** as a white solid (12 mg, 78%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.82 (s, 1H), 8.01 (d, *J* = 7.3 Hz, 2H), 7.75-7.61 (m, 5H), 7.50 – 7.29 (m, 4H), 6.79 (d, *J* = 8.7 Hz, 1H), 4.13 – 3.82 (m, 1H), 3.62 (s, 1H), 3.43 (s, 1H), 3.35 (s, 3H), 3.12 (s, 3H), 2.96 (s, 3H), 2.77 – 2.56 (m, 1H), 2.38 – 2.04 (m, 4H), 1.71 – 1.51 (m, 1H), 1.25 (s, 2H), 1.04 – 0.81 (m, 4H). **IR** (PTFE) ν_{max} = 3284, 3127, 3059, 3032, 2965, 2931, 2819, 1614, 1543, 1491, 1403, 1308, 1249, 1219, 1154, 1095, 1075, 1030, 1007, 911, 855, 814, 733, 698, 646 cm^{-1}. **HRMS** $C_{31}H_{37}N_3O_4$; calculated $(M+H)^+$ = 516.2857; average observed $(M+H)^+$ = 516.2860.

Procedure D: BRD5631 (15 mg, 0.030 mmol) was suspended in DCM (3.0 mL, 0.01 M) and ethylisocyanate (2.84 µL, 0.036 mmol) was added dropwise. The reaction was stirred until complete by LCMS (1 h) and quenched with saturated $NaHCO_3$. The product was extracted with DCM (3x), dried over sodium sulfate, concentrated, and purified by flash chromatography (Isco, silica 4 g, 0-10% MeOH in DCM, 25 min) to give **BRD2443** as a white solid (16.8 mg, 98%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.38 (s, 1H), 7.97 (d, *J* = 7.9 Hz, 2H), 7.64 (dd, *J* = 21.8, 7.4 Hz, 5H), 7.56 – 7.34 (m, 4H), 6.78 (d, *J* = 8.7 Hz, 1H), 5.98 (s, 1H), 4.79 (t, *J* = 9.2 Hz, 1H), 4.66 (d, *J* = 13.7 Hz, 1H), 3.79 – 3.60 (m, 2H), 3.49 (s, 3H), 3.25 – 3.08 (m, 5H), 2.80 (s, 2H), 2.62 (d, *J* = 15.4 Hz, 1H), 1.43 (d, *J* = 6.5 Hz, 2H), 1.25 (s, 4H), 1.11 (t, *J* = 6.9 Hz, 3H), 1.05 (t, *J* = 7.1 Hz, 2H), 1.00 (d, *J* = 6.8 Hz, 3H), 0.91 – 0.73 (m, 1H). **IR** (PTFE) ν_{max} = 3334, 3059, 2967, 2926, 2874, 1626, 1544, 1492, 1461, 1399, 1306, 1251, 1200, 1145, 1096, 1072, 1030, 901, 855, 815, 746, 698, 652, 588 cm^{-1}. **HRMS** $C_{33}H_{40}N_4O_5$; calculated $(M+H)^+$ = 573.3071; average observed $(M+H)^+$ = 573.3066.

Experimental Protocols.

Primary screen for modulators of autophagy. HeLa cells stably expressing GFP-LC3 were plated in 384-well plates (Aurora Biotechnologies, 1022-11330) at 5,000 cells per well in 50 µL IMDM. The following day, 100 nL of compounds were pin-transferred (10 µM) into plates using a CyBi-Well Vario (CyBIO) and incubated with cells for 4 h at 37°C. Cells were then fixed in 3.7% paraformaldehyde (PFA) for 15 min at room temperature, washed, and DNA stained with 2 µg/mL Hoechst 33342 (Sigma, B2261). High-throughput imaging at 20X was performed on an *ImageXpress* Micro automated microscope (Molecular Devices) and the number of GFP punctae per cell was quantified using the *MetaXpress* high-content image analysis software (Transfluor module). Significance was assessed by computing an empirical test statistic, termed *prevalence*, which summarizes the observed shift in mass under the test hypothesis compared to the distribution of null hypothesis. Specifically, it was computed as the area under the curve of the test distribution beyond the critical value corresponding to 95% confidence of the null distribution. The null distribution was estimated from the observed number of autophagic vesicles per cell in DMSO-treated wells.

Dual tagged mCherry-GFP-LC3 reporter assay. HeLa cells stably expressing mCherry-GFP-tagged LC3 were seeded overnight in 384-well plates (Corning, 3712) at 2,000 cells per well in 50 µL IMDM. 400 representative DOS hits along with in-plate controls (PI-103, chloroquine, and bafilomycin A1) were pin-transferred into plates in 8-point dose and incubated at 37°C for 24 h. Cells were then fixed, DNA stained, and imaged as above. *CellProfiler* automated image analysis software was used to quantify the total area of

autophagosomes (mCherry$^+$/GFP$^+$; yellow punctae) and autolysosomes (mCherry$^+$/GFP$^-$; red punctae) per cell (7).

Immunoblotting. *HeLa cells and iBMDMs.* Cells were washed with phosphate buffered saline (PBS) and lysed for 1 h in lysis buffer [100 mM Tris-HCl pH 7.6, 1% NP-40, 100 mM NaCl, and Complete EDTA-free Protease Inhibiter Cocktail (Roche)] on ice. Following SDS-PAGE (TGX Any kD gels, Bio-Rad), proteins were transferred onto PVDF membranes (Immobilon-FL or Immobilon-P, Millipore) and blocked in Odyssey blocking buffer (LI-COR P/N 927) for 1 h at room temperature. Membranes were subjected to overnight primary antibody incubation at 4°C and 1 h secondary antibody incubation at room temperature. Blots in Fig. 2*C*, Fig. S3*E* and Fig. S6 were imaged on the LI-COR infrared imaging system. Blots of phospho-ULK1 and ULK1 in Fig. 2*D* were visualized with SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific). All other blots in Fig. 2*D* were visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). All antibodies and dilutions used are summarized in Table S1.

hESC/hiPSC-derived neuronal cells and MEFs. Cell pellets were lysed on ice in lysis buffer [10 mM Tris-HCl pH 7.4, 2% sodium dodecyl sulfate, 1 mM DTT, 10% glycerol, 120 mg/mL urea, Complete EDTA-free Protease Inhibitor Cocktail (Roche, 11873580001)] for 30 min, boiled for 10 min at 100°C and subjected to SDS–PAGE and immunoblot analysis as previously described (8). Blots were incubated with primary antibodies at 4°C overnight. Immunoblots were then probed with secondary antibodies for 1 h at room temperature, and visualized using Amersham ECL Western Blotting Detection Reagent (GE Healthcare, RPN2106). All antibodies and dilutions used are summarized in Table S1.

CellTiter-Glo assay. HeLa cells were seeded in 384-well plates at 2,000 cells per well in IMDM supplemented with 10% FBS. The following day, 100 nL compounds were pin-transferred into plates using a CyBi-Well Vario (CyBIO) and incubated with cells for 72 h at 37°C. 25 µL of CellTiter-Glo reagent (Promega) was added to each well and incubated for 30 min at room temperature. Plates were analyzed by measuring luminescence with the Synergy H4 plate reader (BioTek).

DQ-BSA assay. HeLa cells were plated overnight in 384-well plates (Corning, 3712) at 2,500 cells per well in 50 µL IMDM. The following day, cells were incubated with 10 µg/mL DQ-BSA (Life Technologies, D12051) for 1 h. Afterward, DQ-BSA was washed away, and cells were subsequently treated with compound for 6 h. In the last 30 min, nuclei were stained with 2 µg/mL Hoechst 33342. Images of live cells were taken by *ImageXpress* Micro microscope using DAPI and Texas Red filters. MetaXpress (Transfluor module) was used to quantify number and intensity of DQ-BSA punctae.

LysoTracker assay. HeLa cells were seeded overnight in 384-well plates (Corning, 3712) at 2,500 cells per well in 50 µL IMDM. The following day, compounds were pin-transferred into plates and incubated at 37°C for 4 h. Subsequently, 10 µL of IMDM containing LysoTracker Red dye (Life Technologies, L7528) and Hoechst 33342 was added to bring the LysoTracker Red dye and Hoechst 33342 to final concentrations of 100 nM and 2 µg/mL, respectively. After staining for 1 h, the media in each well was replaced with PBS and imaging was performed with an *ImageXpress* Micro automated microscope using the DAPI and TRITC filters. MetaXpress (Transfluor module) was used to quantify the number of LysoTracker Red punctae per cell and average intensity of LysoTracker Red per cell.

Quantification of mutant huntingtin aggregates. The percentage of eGFP$^+$ cells with eGFP-HDQ74 aggregates was assessed as described previously (8). Briefly, *Atg5*$^{+/+}$ and *Atg5*$^{-/-}$ MEFs were transfected with 2.5 µg eGFP-HDQ74 construct using FuGENE HD transfection reagent (Promega, E2311) according to the manufacturer's protocol followed by treatment with 10 µM BRD5361 for 48 h, after which the transfected cells were assessed for eGFP-HDQ74 aggregates by fluorescence microscopy.

Differentiation of hESCs/hiPSCs into neuronal and hepatic-like cells. Neurons were generated from neural precursors, which were derived from hESCs and hiPSCs using an embryoid body-based protocol as described previously (9). Differentiation of hESCs and hiPSCs into hepatic-like cells was induced as previously described (10). Generation and maintenance of NPC1 hiPSC-derived neuronal and hepatic-like cultures, which expressed the neuronal (Tuj1 and MAP2) and hepatic (HNF4α and AFP) markers, respectively, were carried out as described previously (2).

Analysis of cell death in hESC/hiPSC-derived neuronal-like cultures. Analysis of cell death in neurons was assessed by DAPI staining and TUNEL labeling (Roche Diagnostics, 11684795910). The TUNEL labeling assay was preformed according to manufacturer's instructions. Fluorescein^{+} nuclei were counted and analyses of DAPI-stained apoptotic nuclear morphology in neuronal cultures were performed as described elsewhere (2).

Analysis of cell death in hESC/hiPSC-derived hepatic-like cultures. Analysis of apoptotic cells in hepatic cultures was performed with FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, 556547) using FACS according to the manufacturer's protocol, as previously described (2).

Quantitative RT-PCR. Cells were seeded in 96-well plates and subjected to compound treatment. Cells were then washed once with PBS, and mRNA was harvested from cells using Dynabeads mRNA DIRECT Kit (Invitrogen, 61012) and converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, 4368813) following manufacturers' instructions. RT-PCR was performed on a CFX384 Real-Time PCR Detection System (Bio-Rad) with TaqMan Fast 2x Master Mix (Life Technologies, 4444557). The following TaqMan probes (Life Technologies) were used: Sqstm1 Mm00448091 and Actb Mm00607939. Reaction conditions consisted of 40 cycles of PCR with 60°C annealing temperatures. Relative mRNA levels are acquired by examining the threshold cycle (C_T) values using the equation:
Normalized p62 mRNA level = $2^{-\Delta\Delta C_T}$. $\Delta\Delta C_T = (C_T \text{ p62} - C_T \text{ actin})_{\text{BRD5631-treated}} - (C_T \text{ p62} - C_T \text{ actin})_{\text{DMSO-treated}}$.

ATG16L1 CRISPR methods. The second exon of *ATG16L1* was targeted in HeLa cells using the px330 plasmid CRSIPR system as described (11). Briefly, a 20-nucleotide guide sequence complementary to exon 2 of *ATG16L1* (CCCTGTCCTT CCGCTGCATT) was cloned into px330 as described. The Cas9 vector containing *ATG16L1*-specific sgRNA sequence was then used to transfect HeLa cells using FuGENE (Roche) according to the manufacturer's instructions. 48-72 h post transfection, cells were plated in limiting dilution in 96-well plates to isolate single cell clones. DNA was isolated from clonal populations using DNeasy (Qiagen) and *ATG16L1* exon 2 was PCR-amplified and sequenced to identify clones with frameshift mutations in all loci. ATG16L1 loss was also confirmed at the protein level. Western blotting for LC3 shift confirmed the absence of LC3-II conversion in these cells.

Atg5 CRISPR method. The second exon of *Atg5* was targeted in iBMDMs using the pXPR_001 plasmid CRISPR system as described (12). Briefly, a guide sequence complementary to exon 2 of *Atg5* (CACCGAAGATGTGCTTCGAGATGTG) was cloned into pXPR_001 plasmid, and co-transfected into HEK293T cell with pCMV-dR8.91 and VSV-G plasmids for lentivirus production. iBMDMs were transduced with lentivirus for 48 h, and then selected with puromycin. After 7-10 days of selection, ATG5 loss in iBMDM populations were confirmed by western blotting for LC3 shift and loss of ATG5-ATG12 complex, and used in experiments immediately once confirmed.

Bioluminescent bacterial replication assay. Bioluminescent bacterial replication assays were performed as previously described (13). Briefly, HeLa cells were seeded overnight in 96-well plates (Corning, 3904) at 1 x 10^4 cells per well in 100 µL antibiotic-free IMDM. The following day, an overnight bacterial culture of *S. enterica* serovar Typhimurium expressing the *Photorhabdus luminescens lux* operon (Perkin Elmer, Xen26) was diluted

1:30 in LB media containing 30 μg/mL kanamycin and cultured with shaking at 37°C for 4 h. The bacterial culture was then diluted 1:40 in antibiotic-free IMDM and incubated with HeLa cells for 30 min at 37°C. Following infection, the bacterial suspension was removed and replaced with IMDM containing 20 μg/mL gentamicin. After 10 min at room temperature, the cells were washed a second time without 10 min incubation, then the media was replaced with IMDM containing compounds and 20 μg/mL gentamicin. Plates were then incubated at 37°C and analyzed at indicated time points post infection by measuring luminescence with the Synergy H4 plate reader (BioTek).

Gentamicin protection assay. HeLa cells were plated at 1 x 10^4 cells per well in 100 μL antibiotic-free IMDM. The following day, cells were infected for 30 min with 1:40 diluted *S.* Typhimurium Xen26 (overnight culture diluted 1:30 and grown for an additional 4 h) in antibiotic-free IMDM media. Cells were washed with media containing 20 μg/mL gentamicin and incubated with media containing the indicated compound and 20 μg/mL gentamicin for 20 h. Cells were then washed, lysed with 1% Triton X-100 in PBS, diluted, and plated on LB 50 μg/mL kanamycin. CFUs were counted after a 20 h incubation at 37°C.

Compound toxicity to *Salmonella*. *S.* Typhimurium Xen26 (overnight culture diluted 1:30 and grown for an additional 4 h) was diluted 1:4000 into antibiotic-free IMDM media and 50 μL of this solution was added to each well of a 96-well plate (Corning, 3904). Bacteria solutions were treated with 50 μL of each compound (20 μM) or gentamicin (20 μg/mL) in IMDM media (final concentrations of 10 μM and 10 μg/mL, respectively). After 12 h at 37°C, plates were analyzed by measuring luminescence with the Synergy H4 plate reader (BioTek) to determine bactericidal activity of compounds in the absence of mammalian cells.

LC3 or NDP52/*Salmonella* colocalization assay. HeLa cells were seeded overnight in 96-well plates (Corning, 3904) at 12,500 cells per well, or on coverslips in 24-well plates at 50,000 cells per well in antibiotic-free IMDM. The following day, the medium was replaced with antibiotic-free IMDM containing compounds. The culture of *S. enterica* serotype Typhimurium strain SL1344 expressing DsRed (Clontech) was prepared as previously described (13). After the 3-h compound pretreatment, antibiotic-free IMDM containing 1:20 diluted bacterial culture was added to each well (25 μL for 96-well plates or 50 μL for 24-well plates). Cells were infected for 20 min at 37°C. Cells were washed three times with IMDM containing 50 μg/mL gentamicin and subsequently incubated in IMDM containing 20 μg/mL gentamicin and compounds. At indicated post-infection time points, the medium was aspirated and cells were fixed in methanol at -20°C for 2.5 min. Cells were then incubated with primary antibodies (rabbit α-LC3B, mouse α-NDP52, and goat α-*Salmonella* CSA1) diluted in 10% donkey serum (Jackson ImmunoResearch) in PBS for 1 h at room temperature. Cells were then washed and incubated with secondary antibodies (Alexa Fluor 488- conjugated donkey α-rabbit IgG, Cy5-conjugated donkey α-mouse IgG, and Alexa Fluor 546-conjugated donkey α-goat IgG) and 2 μg/mL Hoechst 33342 in 10% donkey serum in PBS at room temperature for 1 h. Imaging of cells in 96-well plates was performed with *ImageXpress* Micro automated microscope, with nine 40x images taken per well and with DAPI, GFP, TRITC, and Cy5 filters. *CellProfiler* was used to quantify the number of $LC3^+$ bacteria and $NDP52^+$ bacteria (7). Coverslips were scored under wide-field fluorescence illumination with a 64X lens and a 1.6X Optovar (Zeiss Axio Observer.D1; Carl Zeiss MicroImaging). The number of $LC3^+$ bacteria was scored in randomly chosen fields, with at least 100 bacteria examined on each coverslip, and triplicate coverslips examined in each independent experiment.

IL-1β secretion assay. *Immortalized bone marrow-derived macrophages (iBMDMs).* To generate an iBMDM line, BMDMs were infected with a J2 retrovirus (carrying the v-myc and v-raf oncogenes) and cultured at 37°C in DMEM containing 10% FBS and 100 U/mL penicillin/streptomycin for two weeks with 5 ng/ml M-CSF followed by two weeks without M-CSF. On day 1 of the assay, 1 x 10^5 iBMDMs per well were plated in 96-well plates (Corning, 3904). The following day, cells were treated with compound and 100 ng/mL IFNγ for 16 h

followed by a 24-h stimulation with 10 ng/mL LPS, 10 μg/mL MDP, and compound. The amount of IL-1β released into cell supernatants was then measured by ELISA (BD Biosciences, 559603).

Splenic CD11b$^+$ cells. CD11b$^+$ cells were isolated by positive selection using CD11b microbeads (Miltenyi) and spleens derived from C57BL/6 *Atg16l1*$^{wt/wt}$ and *Atg16l1*$^{T300A/T300A}$ mice. Freshly isolated splenic CD11b$^+$ cells were plated at 1 x 10^5 cells per well in 96-well plates (Corning, 3904) and cultured at 37°C in RPMI containing 10% FBS and 100 U/mL penicillin/streptomycin. Cells were treated for 6 h with 100 ng/mL IFNγ followed by a 24-h stimulation with 2 ng/mL LPS, 10 μg/mL MDP, and 100 ng/mL IFNγ. IFNγ and LPS-MDP-IFN treatments were performed in the presence of compound and the amount of IL-1β released was measured as above.

SYTOX Green assay. Following the removal of supernatant for the IL-1β ELISA, cells remaining in the 96-well plate were washed with phosphate-free buffer and then 50 μL of SYTOX Green solution in phosphate-free buffer (100 nM) (Life Technologies, S7020) was added to each well. After 30 min, the staining solution was removed, the cells were washed twice with phosphate-free buffer, and total fluorescence was measured with the Synergy H4 plate reader [Excitation/Emission (nm) 504/523].

Mice. T300A knock-in mice were generated and maintained as previously described (14). Animals were housed in a pathogen-free facility, and all procedures were performed in accordance with the institutional animal care and use committee at Massachusetts General Hospital.

Statistical analysis. For GFP-LC3 punctae formation assays, dose curves and EC_{50} values were determined using the log (agonist) vs. normalized response – variable slope model in Prism 6 (GraphPad). The chemical moieties enrichment analysis (Fig. S2) was performed in MATLAB R2013b (MathWorks). Densitometry analyses on the immunoblots from multiple experiments were performed by Image J software (NIH) as previously described (8). *P* values of Student's t-test were determined using Prism 6 (GraphPad). **P* < 0.05; ***P* < 0.01; ****P* < 0.001; ns, non-significant.

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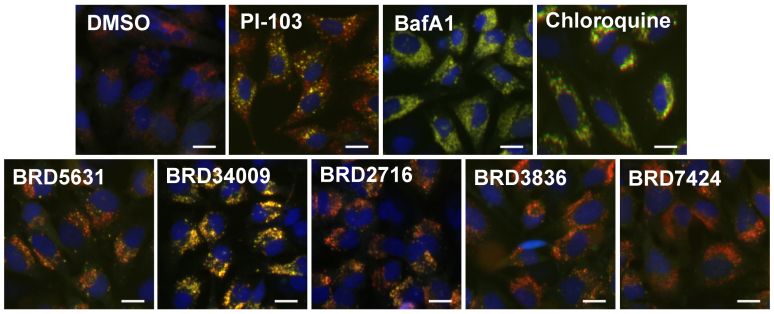

Fig. S1. DOS compounds promote autophagic flux to various extents. HeLa cells stably expressing mCherry-GFP-LC3 were treated with compounds for 24 h, after which cells were fixed and stained with Hoechst 33342 and punctae number was quantified by fluorescence microscopy and automated image analysis. Representative images of DMSO, PI-103 (5 μM), chloroquine (60 μM), bafilomycin A1 (BafA1, 100 nM) and BRD5631, BRD34009, BRD2716, BRD3836, and BRD7424 (each 10 μM) are shown. $GFP^+/mCherry^+$ punctae represent presumed autophagosomes and $GFP^-/mCherry^+$ punctae represent presumed autolysosomes. Scale bars represent 10μm.

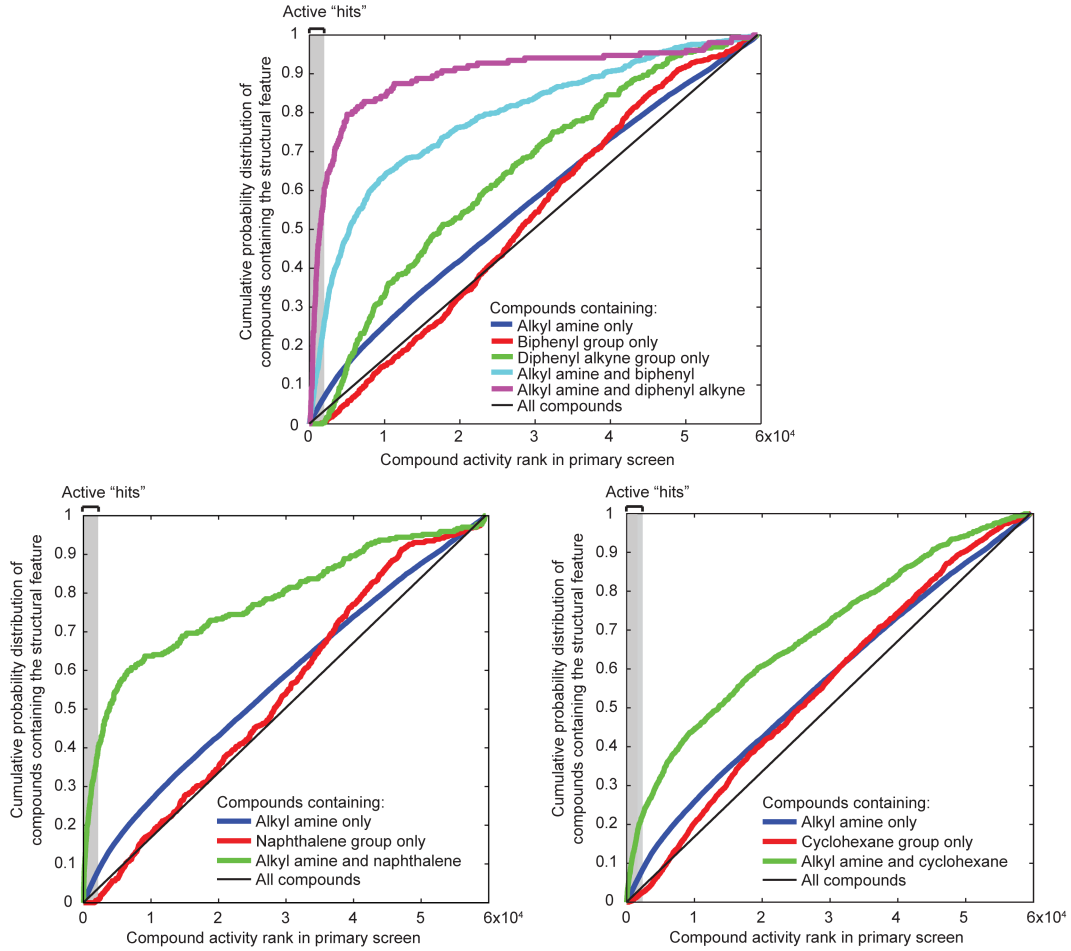

Fig. S2. Chemical moieties are enriched among hits that induce GFP-LC3 punctae formation. 59,541 DOS compounds were ranked based on their activities in the primary screen. Compounds containing different structural features were selected and the cumulative probability distribution of each class was plotted.

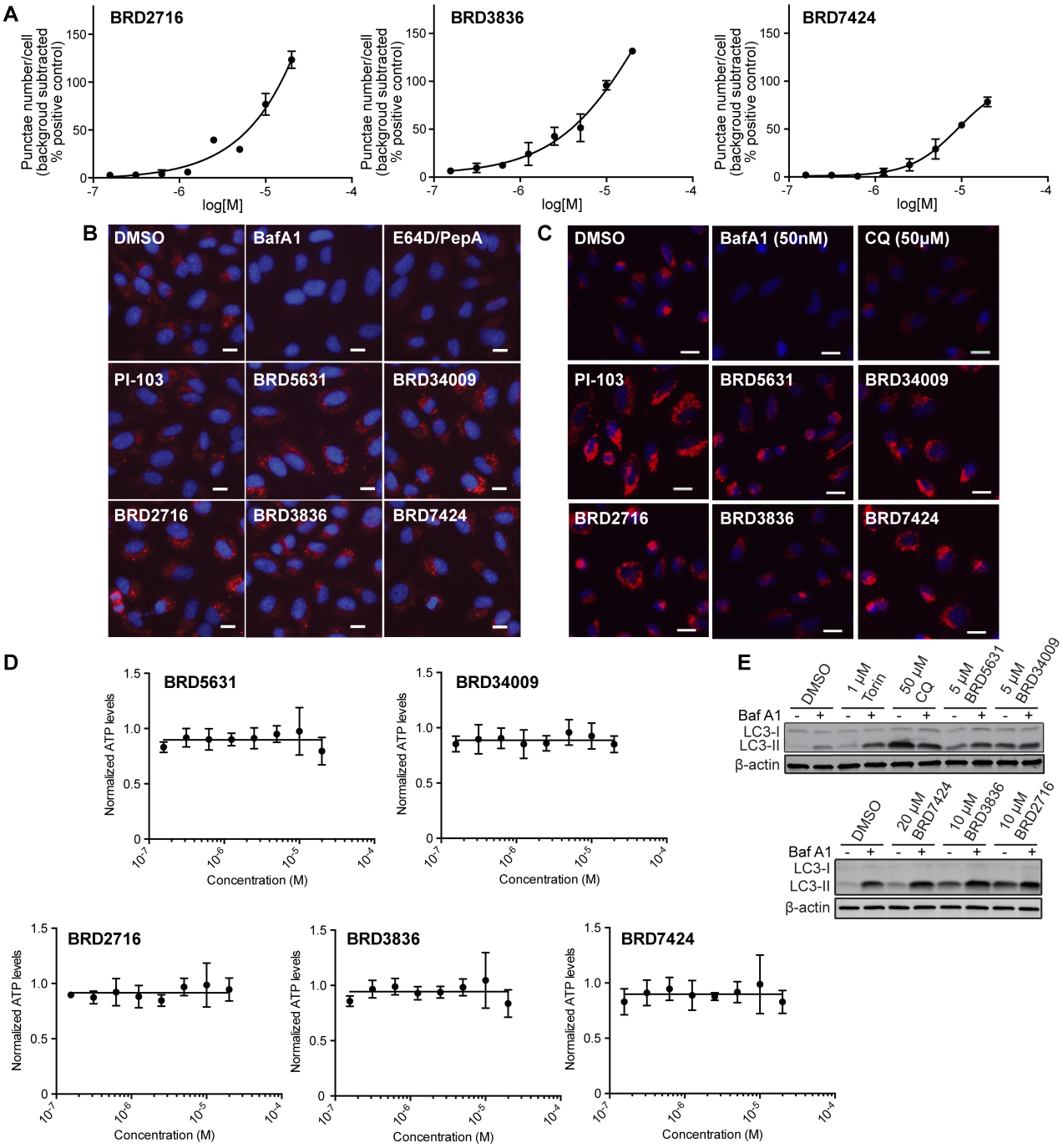

Fig. S3. DOS compounds induce autophagosome formation without perturbing lysosomal functions and without significant toxicity. (*A*) HeLa cells stably expressing GFP-LC3 were treated with compounds in 8-point dose (4 h), after which cells were fixed and stained with Hoechst 33342 and punctae number was quantified by fluorescence microscopy and automated image analysis. Dose curves were generated using "log(agonist) vs. response – variable slope" model in Prism 6. Data points of dose curves are presented as the mean ± SD, n = 3 from a representative experiment. (*B*) HeLa cells were pulsed with DQ-BSA (10 μg/mL) (1 h) followed by a 6-h chase with the indicated compound. In the last 30 min, nuclei were stained with Hoechst 33342, and number and intensity of DQ-BSA punctae were quantified in live cells by fluorescence microscopy and automated image analysis. Representative images are shown. Scale bars represent 10 μm. (*C*) HeLa cells were treated with compounds (4 h), then stained with LysoTracker Red dye (100 nM) and Hoechst 33342 (1 h). Number and intensity of LysoTracker Red punctae were quantified in live cells by fluorescence microscopy and automated image analysis. Representative images are shown. Scale bars represent 10 μm. (*D*) HeLa cells were treated with compounds in 8-point dose for 72 h, after which cell viability was assessed by measuring cellular ATP levels using CellTiter-Glo reagent. ATP levels were normalized to DMSO. Dose curves were generated using "log(agonist) vs. normalized response" model in Prism 6. Data are presented as the mean ± SD, n = 2 from three independent experiments. (*E*) HeLa cells were treated with indicated compounds (7 h). For the last 4 h, either DMSO or BafA1 (100 nM) was added, then protein samples were harvested for immunoblot analysis.

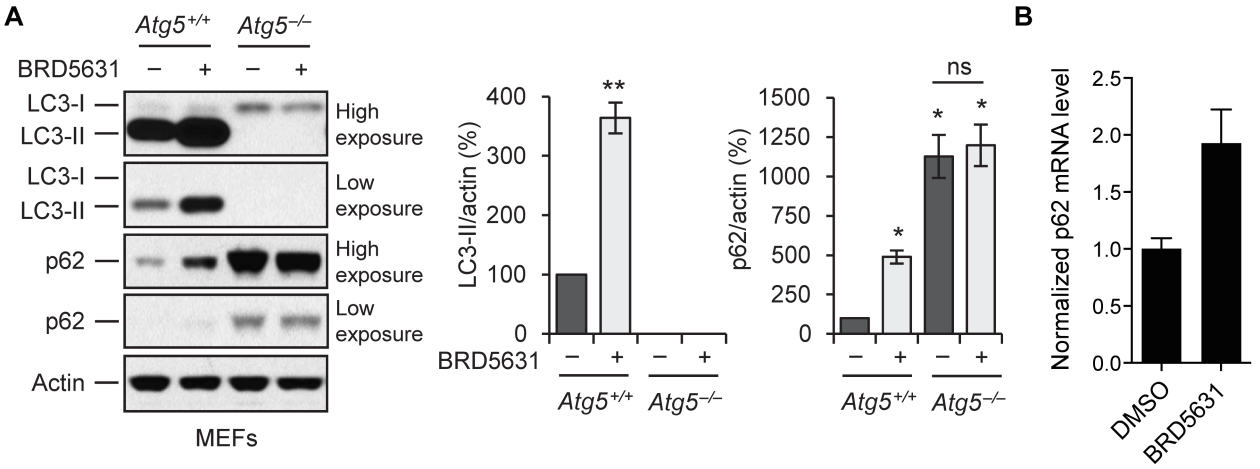

Fig. S4. BRD5631 induces changes in LC3 and p62 levels in MEFs. (*A*) *Atg5*$^{+/+}$ and *Atg5*$^{-/-}$ MEFs, with or without treatment with 10 μM BRD5631 (48 h), were subjected to immunoblot analysis with anti-LC3, anti-p62, and anti-actin antibodies. Densitometric analysis shows LC3-II and p62 levels relative to actin (loading control). Data are presented as the mean ± SD, n = 3 from one representative experiment. *P < 0.05; **P < 0.01; ns = not significant, compared to untreated *Atg5*$^{+/+}$ MEFs, unless indicated otherwise, unpaired Student *t* test. (*B*) *Atg5*$^{+/+}$ MEFs were treated with DMSO or 10 μM BRD5631 (48 h) and subjected to quantitative RT-PCR analysis to measure p62 mRNA levels relative to actin mRNA levels in each well. Results are normalized to DMSO. Data are presented as the mean ± SD, n = 3 from a representative experiment.

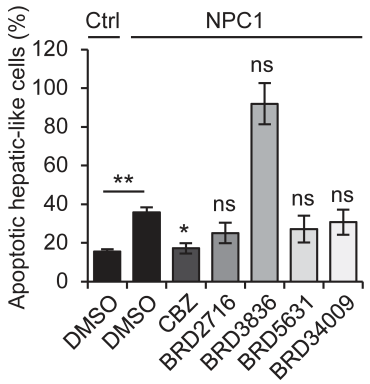

Fig. S5. DOS compounds do not significantly rescue cell death in hepatic-like cells. Control (WIBR3-derived) hepatic-like cells were treated with DMSO (vehicle control), and NPC1 hiPSC (WIBR-IPS-NPC1)-derived hepatic-like cells were treated with DMSO, BRD2716, BRD3836, BRD5631, BRD34009 (10 μM each), or carbamazepine (CBZ, 100 μM, positive control) for 3 days. The FITC Annexin V Apoptosis Detection Kit I by FACS was used to quantify the percentage of apoptotic cells. Results are presented as mean ± SD and are representative of two independent experiments performed in multiple replicates. *P < 0.05; **P < 0.01; ns = not significant, compared to DMSO-treated NPC1 cells unless indicated otherwise, unpaired Student *t* test.

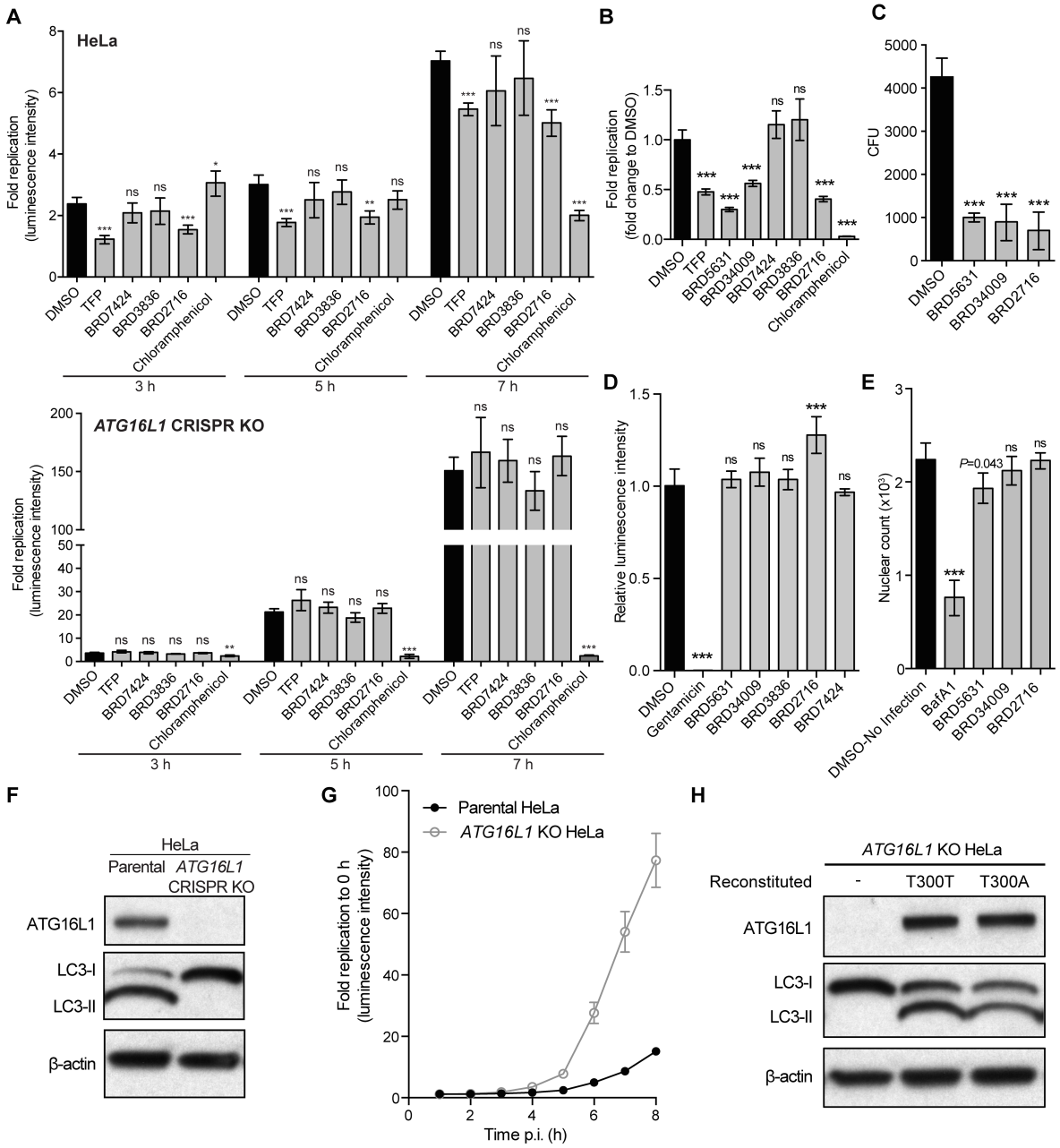

Fig. S6. A subset of DOS compounds promotes *Salmonella* clearance in an autophagy-dependent manner. (*A*-*B*) *Salmonella* survival in cells was assessed in a bioluminescent bacterial replication assay. *Salmonella* luminescent units in live cells were measured at indicated time points in panel *A*, or 20 h postinfection (p.i.) in panel *B*. Fold replication value was calculated for each well as raw luminescence value at each time point divided by the value at 1 h post infection. Data are presented as mean ± SD, n =4 from a representative experiment. (*C*) HeLa cells were infected and washed as above. Cells were then incubated with compounds (20 h) before being lysed. Lysates were plated on LB plates containing kanamycin. Colonies were scored after an overnight incubation. Data are presented as mean ± SD, n = 4 from a representative experiment. (*D*) Xen26 *Salmonella* were incubated with compounds (DMSO, BRD5631, BRD34009, BRD2716, BRD3836, BRD7424 at 10 μM; gentamicin at 10 μg/mL) in mammalian cell growth medium (12 h) and luminescence intensity was measured. Data are presented as mean ± SD, n =4 from a representative experiment. (*E*) HeLa cells were infected with DsRed *Salmonella* Typhimurium (20 min). Cells were then washed and incubated with compounds (DMSO, BRD5631, BRD34009, or BRD2716 at 10 μM; BafA1 at 200 nM). After 20 h, cells were fixed and stained with Hoechst 33342 and the nuclei were counted by fluorescence microscopy. Data are presented as mean ± SD, n = 4 from a representative experiment. (*F*) and (*H*) Cells were subject to immunoblot analysis with anti-ATG16L1, anti-LC3, and anti-β-actin antibodies. (G) *Salmonella* survival in parental HeLa or *ATG16L1* CRISPR KO cells was assessed in a bioluminescent bacterial replication assay. *Salmonella* luminescent units in live cells were measured at indicated time points. Fold replication value was calculated as described above. Data are presented as mean ± SD, n = 4 from a representative experiment. Unpaired Student *t* test was conducted to compare each compound to DMSO at each time point. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; ns = not significant.

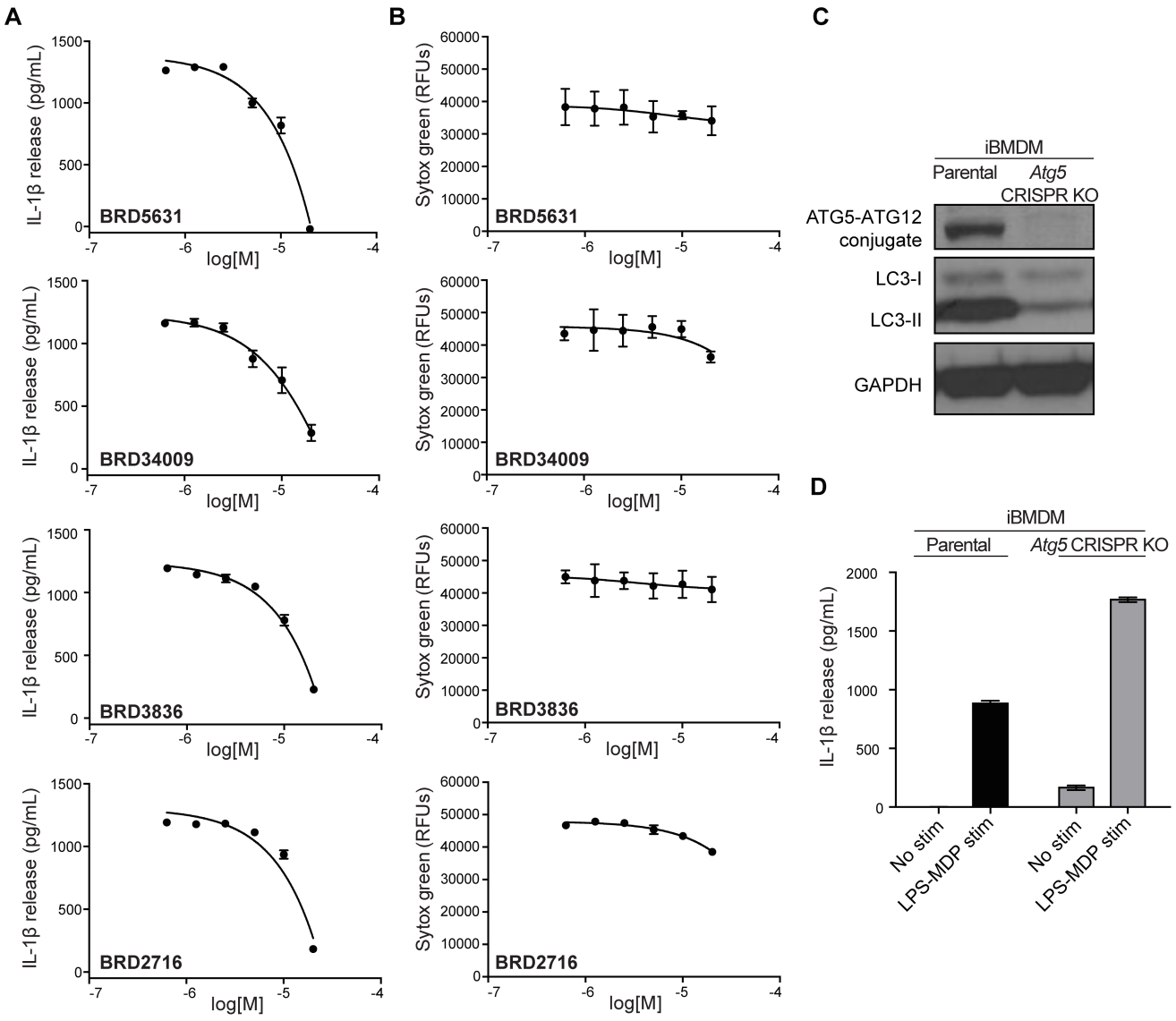

Fig. S7. Autophagy modulators suppress IL-1β secretion in an autophagy-dependent manner. (*A*) IL-1β secretion was measured in immortalized murine BMDMs (iBMDMs) primed with IFNγ (100 ng/mL) (16 h) and stimulated with LPS (10 ng/mL)-MDP (10 μg/mL) (24 h), both in the presence of compounds. The amount of IL-1β released into supernatants was assessed by ELISA. Data are presented as mean ± SD, n = 3 from a representative experiment. Dose curves were generated using "log(inhibitor) vs. response" model in Prism 6. (*B*) iBMDMs were primed, stimulated, and treated with compound as indicated above. After supernatants were removed, cell viability in each well was assessed using SYTOX Green Nucleic Acid Stain following the manufacturer's protocol. Data are presented as mean ± SD, n = 3 from the same representative experiment in panel (*A*). Dose curves were generated using "log(inhibitor) vs. response" model in Prism 6. (*C*) Wild type and *Atg5* CRISPR KO iBMDMs were subject to immunoblot analysis with anti-ATG5, anti-LC3 and anti-GAPDH antibodies. (*D*) Wild type and *Atg5* CRISPR KO iBMDMs were primed with IFNγ (100 ng/mL) (16 h) and stimulated with LPS (10 ng/mL)-MDP (10 μg/mL) (24 h). The amount of IL-1β released into supernatants was measured by ELISA. Data are presented as mean ± SD, n = 3 from a representative experiment.

Table S1. Antibodies used for immunoblotting.

Antibody	Cell type	Vendor	Catalog number	Dilution
Primary				
ATG16L1	HeLa	Cell Signaling	8089	1:1,000
ATG5	iBMDM	Novus Biologicals	NB110-53818	1:1,000
GAPDH	iBMDM	Cell Signaling	5174	1:5,000
GAPDH	ihESC-derived neuronal cells	Santa Cruz Biotechnology	sc-47724	1:5,000
LC3B	HeLa and iBMDM	Sigma Aldrich	L7543	1:1,000
LC3B	MEF	Novus Biologicals	NB100-2220	1:4,000
p62	ihESC-derived neuronal cells	BD Biosciences	610832	1:1,000
p62	MEF	Progen Biotechnik	GP62-C	1:1,000
p70 S6K1	HeLa	Cell Signaling	2708	1:1,000
phospho-p70 S6K1	HeLa	Cell Signaling	9205	1:1,000
phospho-ULK1	HeLa	Cell Signaling	6888	1:500
ULK1	HeLa	Cell Signaling	8054	1:500
β actin	HeLa	Sigma Aldrich	A1978	1:5,000
Actin	MEF	Sigma Aldrich	A2066	1:4,000
Secondary				
Guinea pig IgG (H+L)-HRP	MEF	Abcam	ab50210	1:10,000
Mouse IgG (H+L)-HRP	ihESC-derived neuronal cells	EMD Biosciences	401253	1:10,000
Mouse IgG (H+L)-IRDye 800CW	HeLa	LI-COR Biosciences	926-32210	1:5,000
Mouse Immunoglobulins-HRP	HeLa (in Fig. 2D)	Dako	P0447	1:5,000
Rabbit IgG (H+L)-HRP	MEF	EMD Biosciences	401393	1:10,000
Rabbit IgG (H+L)-IRDye 680LT	HeLa	LI-COR Biosciences	926-68021	1:20,000
Rabbit Immunoglobulins-HRP	HeLa, iBMDM	Dako	P0448	1:5,000