Materials and methods

Synthesis of compound Tert-butyl 4-(2-bromophenyl) piperazine-1-carboxylate (compound S1). To a solution of 1,2-dibromobenzene (236 mg, 1 mmol) and *tert*-butyl piperazine-1-carboxylate (186 mg, 1 mmol) in toluene (2.5 ml) was added 1,1'-binaphthyl-2,2'-bis(diphenylphosphine) (47 mg, 0.075 mmol), potassium *tert*-butoxide (135 mg, 1.2 mmol) and tris(dibenzylideneacetone)dipalladium (23 mg, 0.025 mmol). The mixture was stirred for 24 h at 80°C under argon atmosphere. The solution was diluted with ethyl acetate (EtOAc) (20 ml) and extracted with H_2O (20 ml). The organic layer was washed with brine (20 ml), dried over sodium sulfate, filtered and concentrated under reduced pressure to afford the crude product, which was purified by silica gel chromatography [petroleum ether (PE)/EtOAc=10:1] to give the desired product (201 mg, 58%) as a red oil (Fig. S2). The physical state was red oil. Thin layer chromatography (TLC): R_f=0.52 (PE/EtOAc=8:1). Proton nuclear magnetic resonance spectroscopy (^{1}H NMR) [400 MHz, deuterated chloroform ($CDCl_3$)] δ 7.58 (d, *J*=7.9 Hz, 1H), 7.29 (d, *J*=7.8 Hz, 1H), 7.03 (d, *J*=8.0 Hz, 1H), 6.96-6.91 (m, 1H), 3.61 (t, *J*=5.0 Hz, 4H), 2.98 (t, *J*=4.9 Hz, 4H), 1.50 (s, 9H) (Fig. S7). ^{13}C NMR (101 MHz, $CDCl_3$) δ 154.82, 150.39, 133.84, 128.31, 124.67, 121.02, 120.04, 79.71, 51.63, 44.39, 28.48 (Fig. S8). High-resolution mass spectrometry (HRMS) (ESI): calculated for $C_{15}H_{21}BrN_2O_2$ $[M+H]^+$ m/z 341.0859, found 341.0885.

Synthesis of compound T Tert-butyl4-(2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)piperazine-1-carboxylate (compound S2). To a solution of S1 (314 mg, 0.92 mmol) and bis(pinacolato)diboron (350 mg, 1.38 mmol) in 1,4-dioxane (5 ml) was added potassium acetate (271 mg, 2.76 mmol) and 1,1'-bis(diphenylphosphino)ferrocene]dichloropalladium(II) (38 mg, 0.046 mmol). The mixture was stirred for 24 h at 80°C under argon atmosphere. The solution was diluted with dichloromethane (CH_2Cl_2) (25 ml) and extracted with H_2O (20 ml). The organic layer was washed with brine (20 ml), dried (Na_2SO_4), filtered and concentrated under reduced pressure to afford the crude product, which was purified by silica gel chromatography (PE/EtOAc=8:1 to PE/EtOAc=4:1) to give the desired product (178 mg, 50%) as a red oil (Fig. S3). The physical state was red oil. TLC: R_f=0.52 (PE/EtOAc=4:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.73 (d, *J*=7.0 Hz, 1H), 7.36 (t, *J*=7.4 Hz, 2H), 6.98 (t, *J*=6.6 Hz, 2H), 6.91 (d, *J* = 8.0 Hz, 1H), 3.66-3.56 (m, 4H), 3.07-2.93 (m, 4H), 1.49 (s, 9H), 1.35 (s, 12H) (Fig. S9). ^{13}C NMR (101 MHz, $CDCl_3$) δ 157.93, 154.87, 137.09, 131.90, 121.74, 117.08, 83.53, 79.64, 53.54, 44.43, 28.49, 24.89 (Fig. S10). HRMS (ESI): calculated for $C_{21}H_{33}BN_2O_4$ $[M+H]^+$ m/z 389.2606, found 389.2629.

Synthesis of compound 3-Bromo-N-(3,4-dichlorobenzyl) benzamide (compound S3). To a solution of 3-bromobenzoic acid (600 mg, 3.0 mmol) and (3,4-dichlorophenyl)methanamine (570 mg, 3.3 mmol) in CH_2Cl_2 (10 ml) was added 1-hydroxybenzotriazole (480 mg,3.6 mmol), triethylamine (900 mg, 9.0 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (690 mg, 3.6 mmol). The reaction mixture was stirred under room temperature (r.t.) for 8 h. The solution was diluted with EtOAc (20 ml) and extracted with saturated sodium bicarbonate (15 ml). The organic layer was washed with brine (15 ml), dried (Na_2SO_4), filtered and concentrated under reduced pressure to afford the crude product, which was purified by chromatography on silica gel (PE/EtOAc=6:1 to PE/EtOAc=4:1) to give the desired product (754 mg, 70%) as a yellow solid (Fig. S4). The physical state was yellow solid. TLC: R_f=0.65 (PE/EtOAc=4:1). Melting Point: 88.2-88.7°C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.92 (s, 1H), 7.70 (d, *J*=7.5 Hz, 1H), 7.64 (d, *J*=7.8 Hz, 1H), 7.41 (d, *J*=8.6 Hz, 2H), 7.31 (t, *J*=7.6 Hz, 1H), 7.18 (d, *J*=8.0 Hz, 1H), 6.52 (s, 1H), 4.58 (d, *J*=5.3 Hz, 2H) (Fig. S11). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.18, 138.25, 135.85, 134.77, 132.76, 131.68, 130.69, 130.23, 129.64, 127.09, 125.61, 122.85, 43.02 (Fig. S12). HRMS (ESI): calculated for $C_{14}H_{10}BrCl_2NO$ $[M-H]^+$ m/z 355.9250, found 355.9255.

Synthesis of compound N-(3,4-dichlorobenzyl)-2'-(piperazin-1-yl)-[1,1'-biphenyl]-3-carboxamide (compound FZU-0045-053). Step 1: To a solution of S2 (380 mg, 0.98 mmol) and S3 (233 mg, 0.65 mmol) in 1,4-dioxane/H_2O (3:1, 4 ml) was added sodium carbonate (207 mg, 1.95 mmol) and tetrakis(triphenylphosphine)palladium(0) (75 mg, 0.07 mmol). The mixture was stirred for 8 h at 80°C under argon atmosphere and then cooled to room temperature. Silica gel (0.5 g) was added to the solution and the solvent was removed under reduced pressure. The crude product was purified by chromatography on silica gel (PE/EtOAc=8:1 to PE/EtOAc=4:1) to give the product S4 (210 mg, 60%) as a white gum (Fig. S5).

Step 2: To a solution of S4 (155 mg, 0.29 mmol) in methanol (MeOH) (2.5 ml) was added 4 M HCl in dioxane (0.7 ml, 2.90 mmol) at 0°C. The mixture was stirred under r.t. for 30 min, and the solvent was removed under reduced pressure. The crude product was diluted with CH_2Cl_2/MeOH/$NH_3{\cdot}H_2O$ (100:10:1, 2 ml) and then the solvent was removed under vacuum. The crude product was purified by chromatography on silica gel (CH_2Cl_2/MeOH/$NH_3{\cdot}H_2O$=100:10:1) to give the desired product (85 mg, 66%) as a white gum (Fig. S5). The physical state was white gum. TLC: R_f=0.51 (CH_2Cl_2/MeOH/$NH_3{\cdot}H_2O$=100:10:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.11 (s, 1H), 7.80 (d, *J*=7.5 Hz, 1H), 7.73 (d, *J*=7.6 Hz, 1H), 7.48-7.41 (m, 2H), 7.38 (d, *J*=8.1 Hz, 1H), 7.32-7.27 (m, 1H), 7.23 (d, *J*=7.5 Hz, 1H), 7.17 (d, *J*=8.1 Hz, 1H), 7.08 (d, *J*=7.3 Hz, 1H), 7.03 (d, *J*=8.5 Hz, 1H), 6.85-6.78 (m, 1H), 4.58 (d, *J*=5.6 Hz, 2H), 2.79-2.73 (m, 4H), 2.72-2.67 (m, 4H) (Fig. S13). ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.56, 150.63, 141.60, 138.80, 133.86, 132.69, 132.31, 131.50, 131.26, 130.64, 129.59, 128.91, 128.59, 127.46, 127.13, 125.51, 122.99, 118.67, 52.34, 46.06, 42.89 (Fig. S14). HRMS (ESI): calculated for $C_{24}H_{23}Cl_2N_3O$ $[M+H]^+$ m/z 440.1291, found 440.1292.

Synthesis scheme for FZU-0045-053. Compound FZU-0045-053 was an intermediate in the synthesis of anti-tumor drug LLY-507 derivatives. It was synthesized by a series of reactions of 1,2-dibromobenzene and tert-butyl piperazine-1-carboxylate (Fig. S6).

Figure S1. Preliminary identification that compound 053 regulates autophagy. (A) Untransfected WT MDA-MB-231 cells were used as the control. After establishing a stable transfected cell line by transfecting MDA-MB-231 cells with pGMLV-CMV-RFP-GFP-hLC3-Puro lentivirus and applying puromycin selection, the cells were treated with RAPA (10 μM) for 24 h to induce autophagy. Red and green fluorescent signals in MDA-MB-231 GFP/RFP-LC3B cells were then captured under a fluorescence microscope. Scale bar, 20 μm. (B) Untransfected WT MCF-7 cells were used as the control. After establishing a stable transfected cell line by transfecting MCF-7 cells with pGMLV-CMV-RFP-GFP-hLC3-Puro lentivirus and applying puromycin selection, the cells were treated with RAPA (10 μM) for 24 h to induce autophagy. Red and green fluorescent signals in MCF-7 GFP/RFP-LC3B cells were then captured under a fluorescence microscope. Scale bar, 20 μm. (C) MCF-7 cells transfected with mRFP-GFP-LC3 virus were treated with 053 (5 μM) for 24 h, fluorescence were observed under an inverted fluorescence microscope. Scale bar, 40 μm. (D) ImageJ (1.8.0.1) software was used to analyze the number of fluorescent spots. Data are presented as mean ± SEM (n=5). Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple comparisons test to compare all experimental groups against a single control group. ****P<0.0001 vs. Control. 053, FZU-0045-053; LC3B, microtubule-associated protein 1A/1B-light chain 3B; CQ, chloroquine; RAPA, rapamycin.

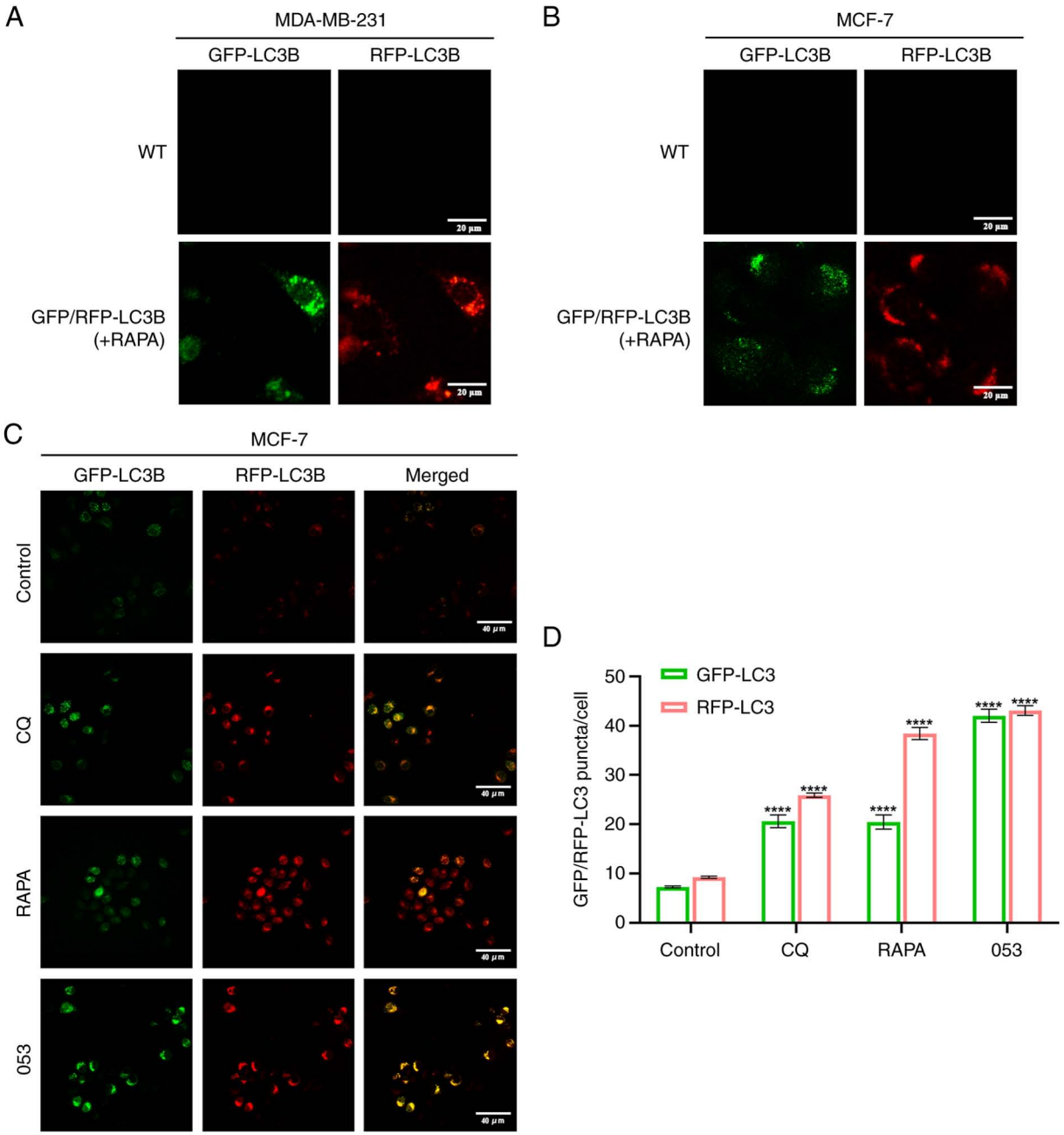


Figure S2. Synthesis of compound Tert-butyl 4-(2-bromophenyl)piperazine-1-carboxylate (compound S1). Boc, Tert-butyloxycarbonyl; 1-Boc-piperazine, Tert-butyl piperazine-1-carboxylate; $Pd_2(dba)_3$, Tris(dibenzylideneacetone)dipalladium; BINAP, 1,1'-Binaphthyl-2,2'-bis(diphenylphosphine).

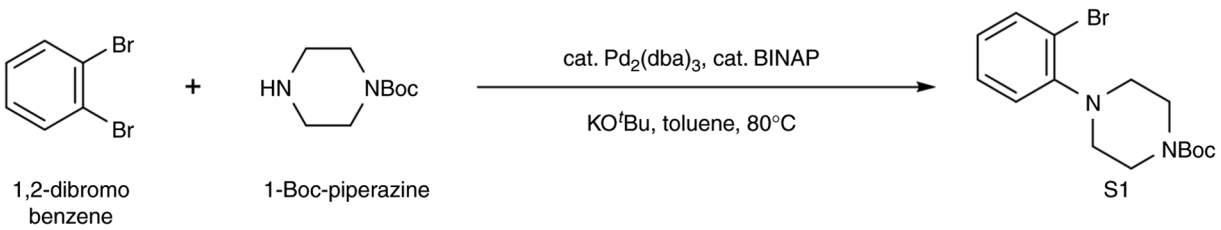
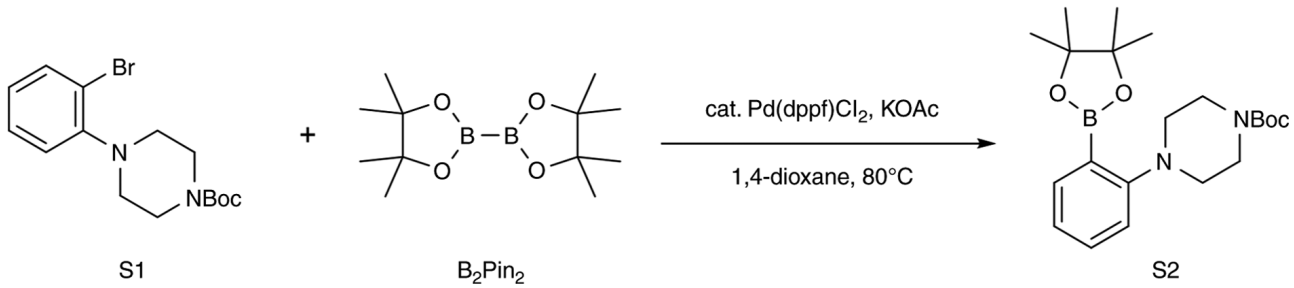
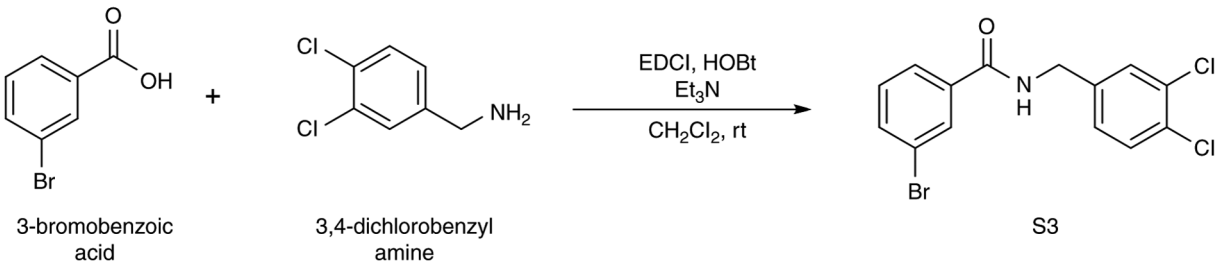


Figure S3. Synthesis of compound Tert-butyl4-(2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)piperazine-1-carboxylate (compound S2). B2pin2, Bis(pinacolato)boron; Pd(dppf)Cl_2, [1,1'-Bis(diphenylphosphino)ferrocene]dichloropalladium(II).



S1 B_2Pin_2 S2

Figure S4. Synthesis of compound 3-Bromo-N-(3,4-dichlorobenzyl)benzamide (compound S3). EDCI, 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; HOBt, 1-hydroxybenzotriazole; Et_3N, triethylamine; CH_2Cl_2, dichloromethane; rt, room temperature.

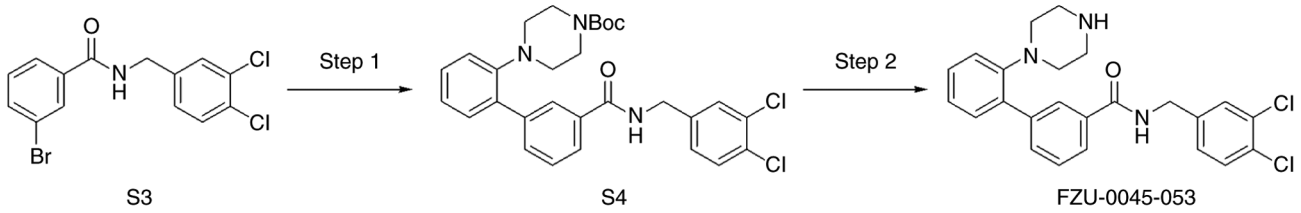


3-bromobenzoic acid

3,4-dichlorobenzyl amine

S3

Figure S5. Synthesis of compound N-(3,4-dichlorobenzyl)-2'-(piperazin-1-yl)-[1,1'-biphenyl]-3-carboxamide (compound FZU-0045-053).



S3 S4 FZU-0045-053

Figure S6. Synthesis scheme for FZU-0045-053. (A) Pd2(dba)3, BINAP, KOtBu, toluene, 80°C, 58%; (B) B_2Pin_2, Pd(dppf)Cl_2, KOAc, 1,4-dioxane, 80°C, 50%; (C) 3,4-dichlorobenzylamine, EDCI, HOBt, Et_3N, CH_2Cl_2, 25°C, 70%; (D) (i) S2, 1,4-dioxane/H_2O (3:1), Na_2CO_3, Pd$(PPh_3)_4$, 80°C, 60%; (ii) HCl in dioxane, MeOH, 25°C, 66%. 053, FZU-0045-053; B_2pin_2, Bis(pinacolato) boron; Pd(dppf)Cl_2, [1,1'-Bis(diphenylphosphino)ferrocene]dichloropalladium(II); EDCI, 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; HOBt, 1-hydroxybenzotriazole; Et3N, triethylamine; CH_2Cl_2, dichloromethane; BINAP, 1,1'-Binaphthyl-2,2'-bis(diphenylphosphine); KOtBu, potassium tert-butoxide; KOAc, potassium acetate; MeOH, methanol; Pd$(PPh_3)_4$, Tetrakis(triphenylphosphine)palladium(0).

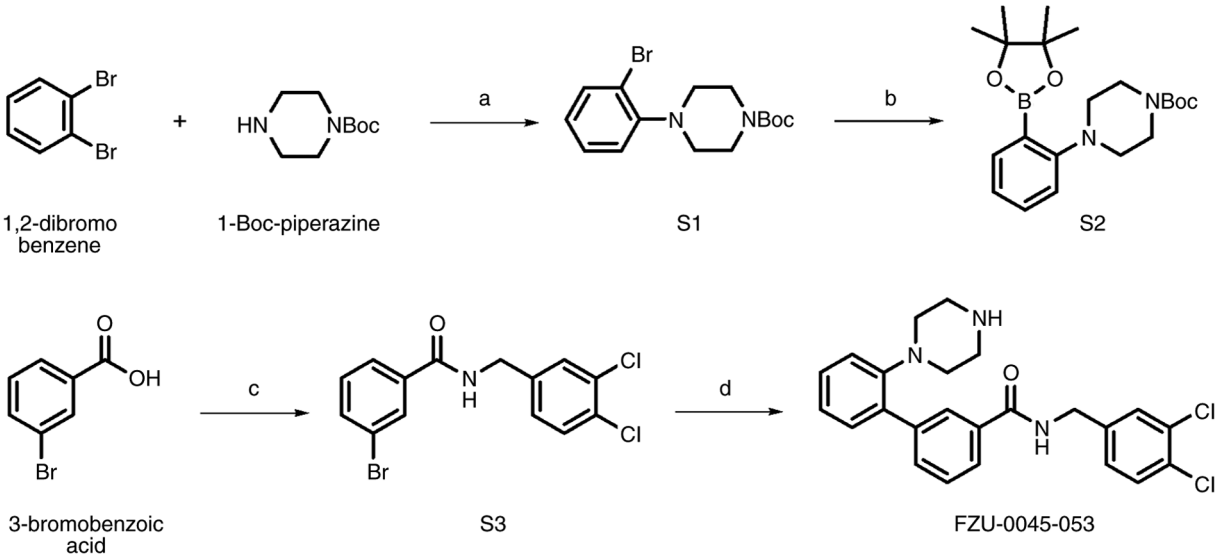


Figure S7. ^{1}H NMR Spectra of compound S1.

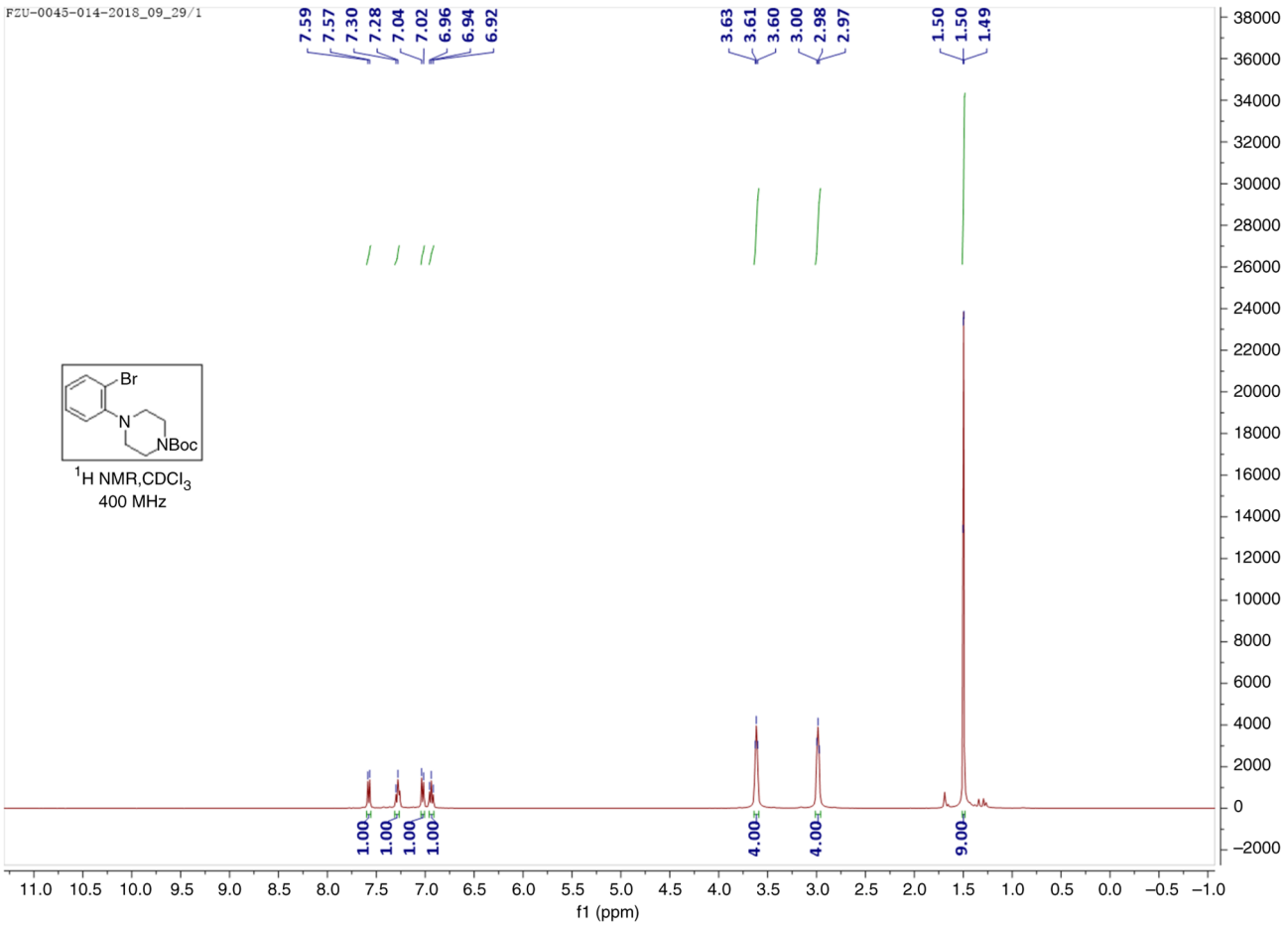


Figure S8. ^{13}C NMR Spectra of compound S1.

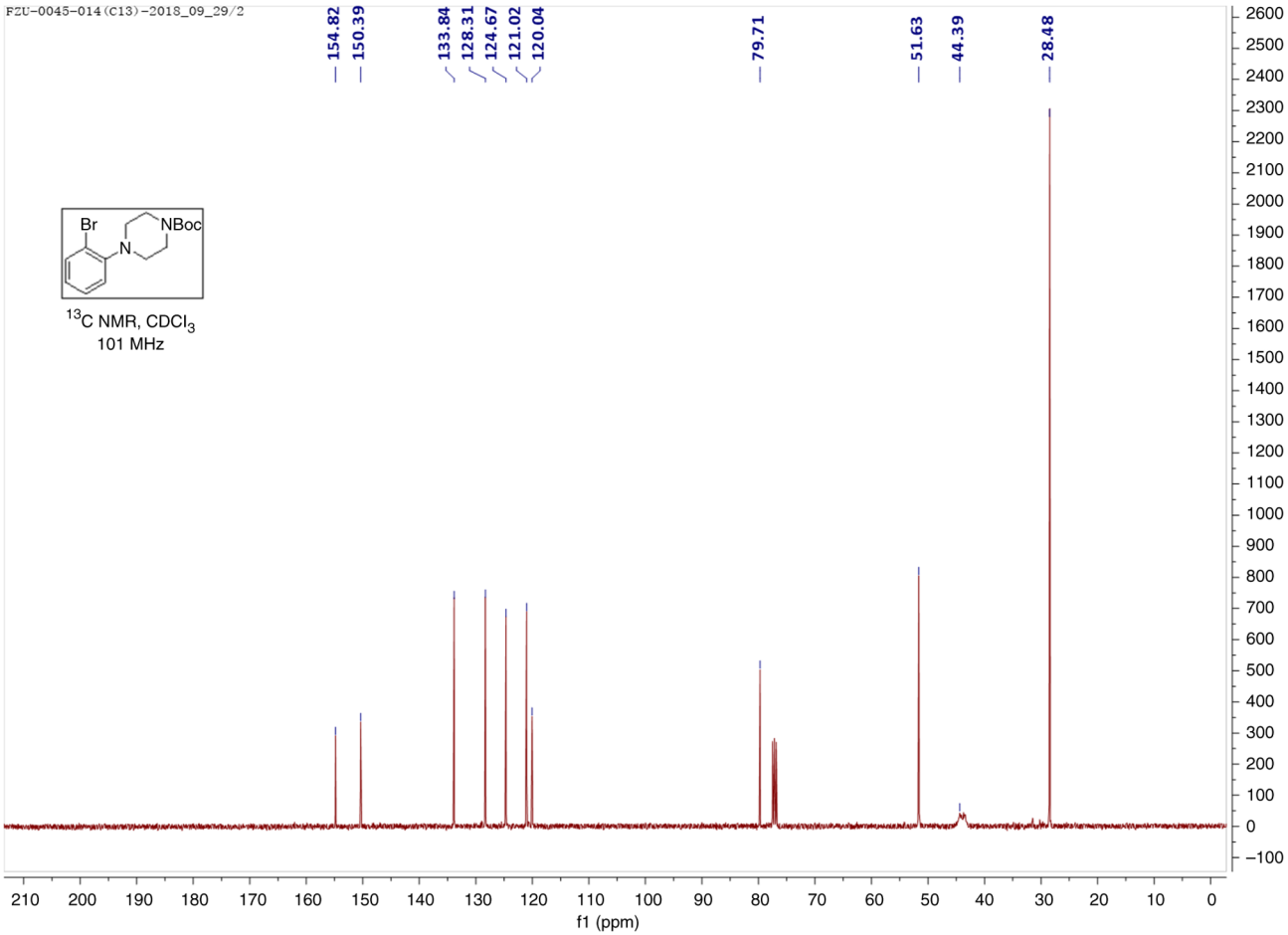


Figure S9. ^{1}H NMR Spectra of compound S2.

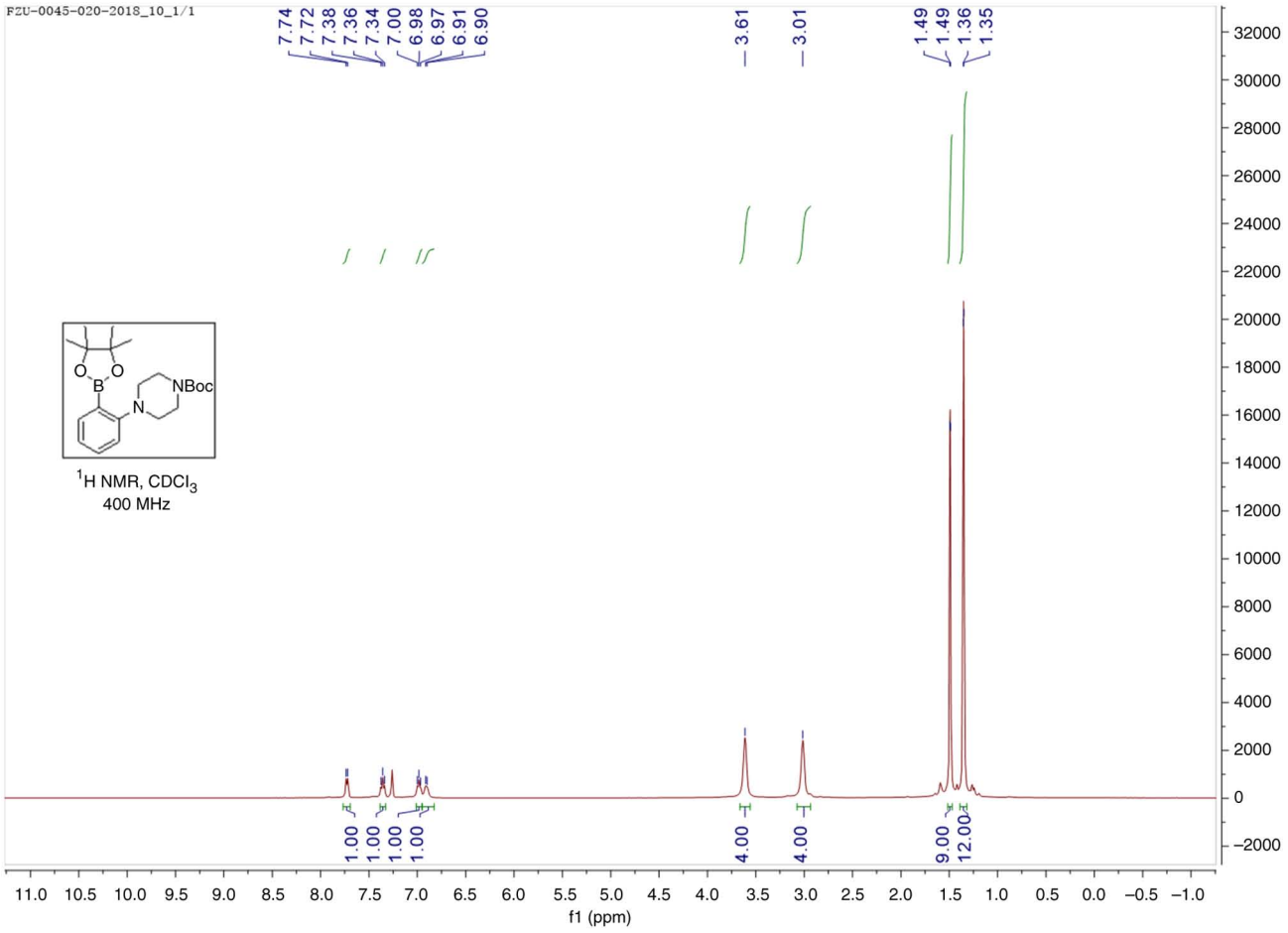


Figure S10. ^{13}C NMR Spectra of compound S2.

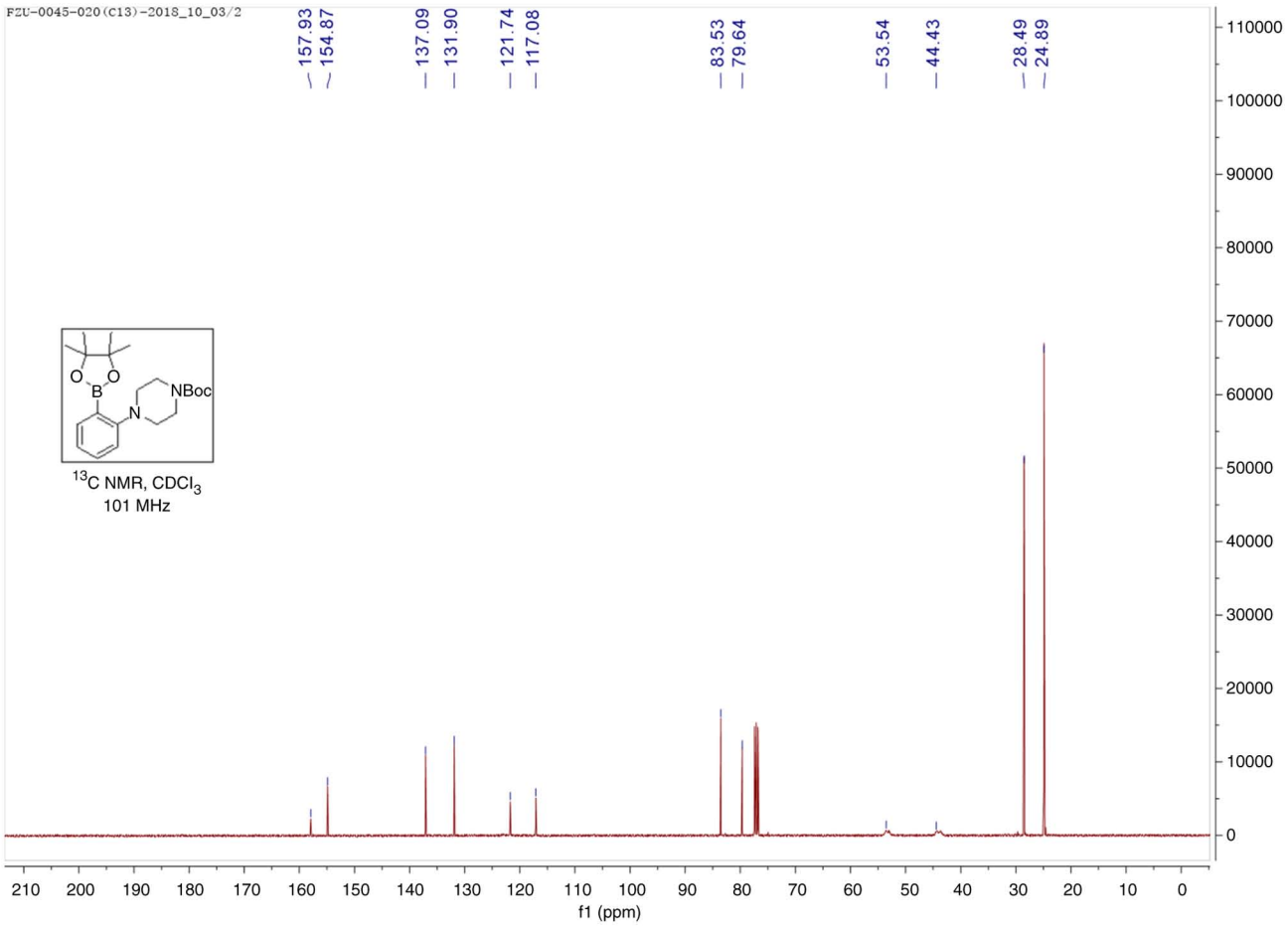


Figure S11. ^{1}H NMR Spectra of compound S3.

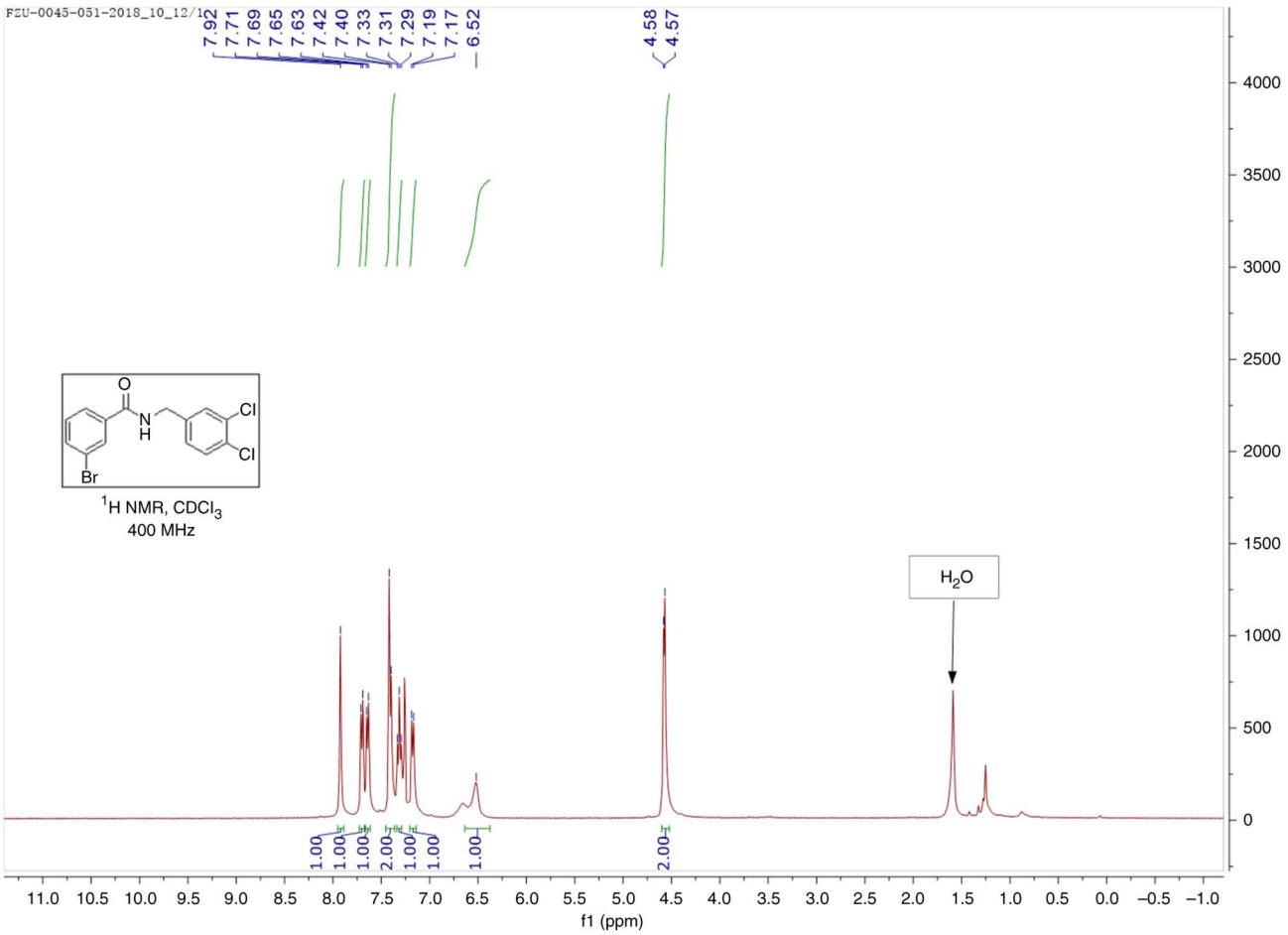


Figure S12. ^{13}C NMR Spectra of compound S3.

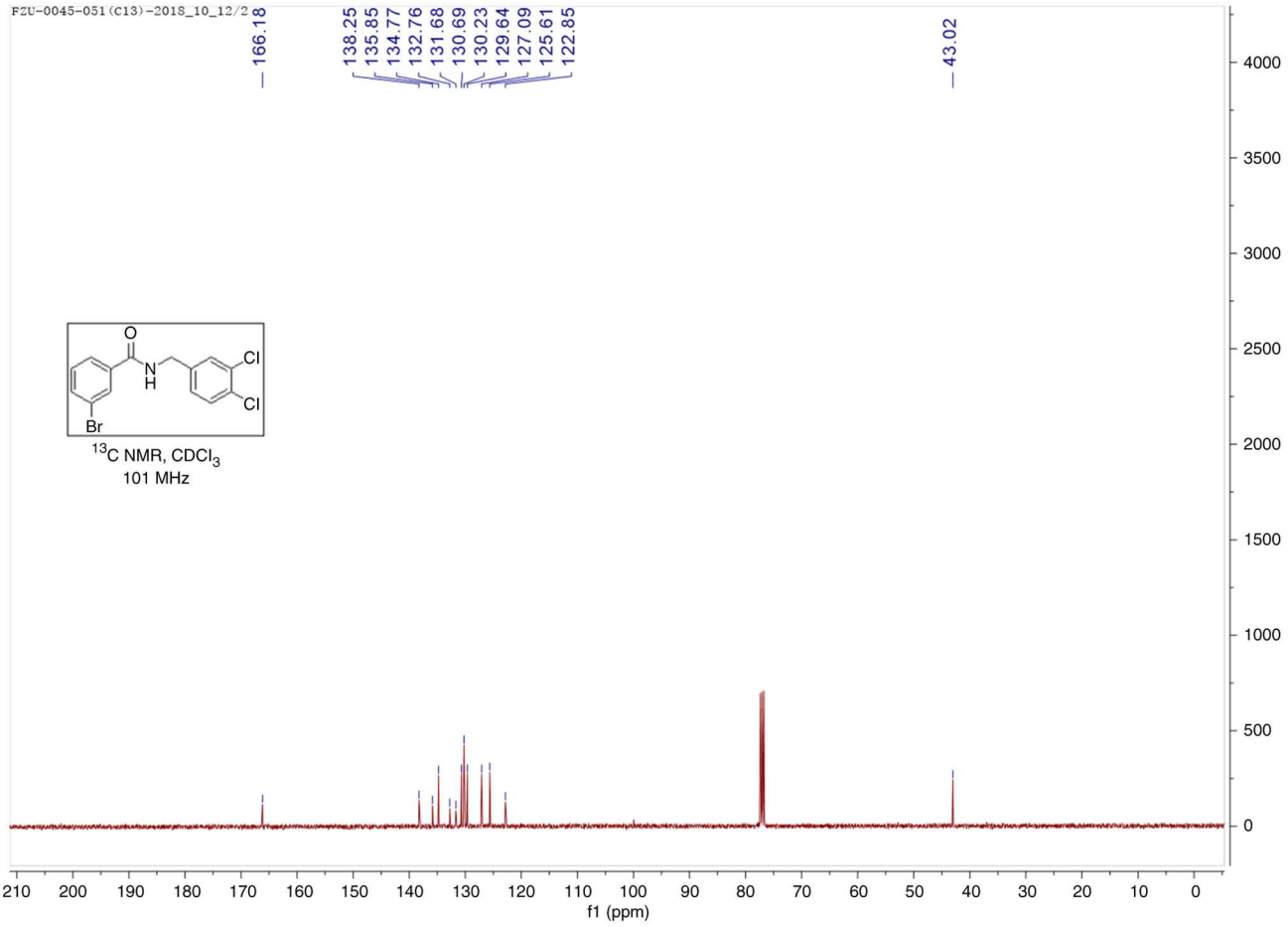


Figure S13. ^{1}H NMR Spectra of compound 053.

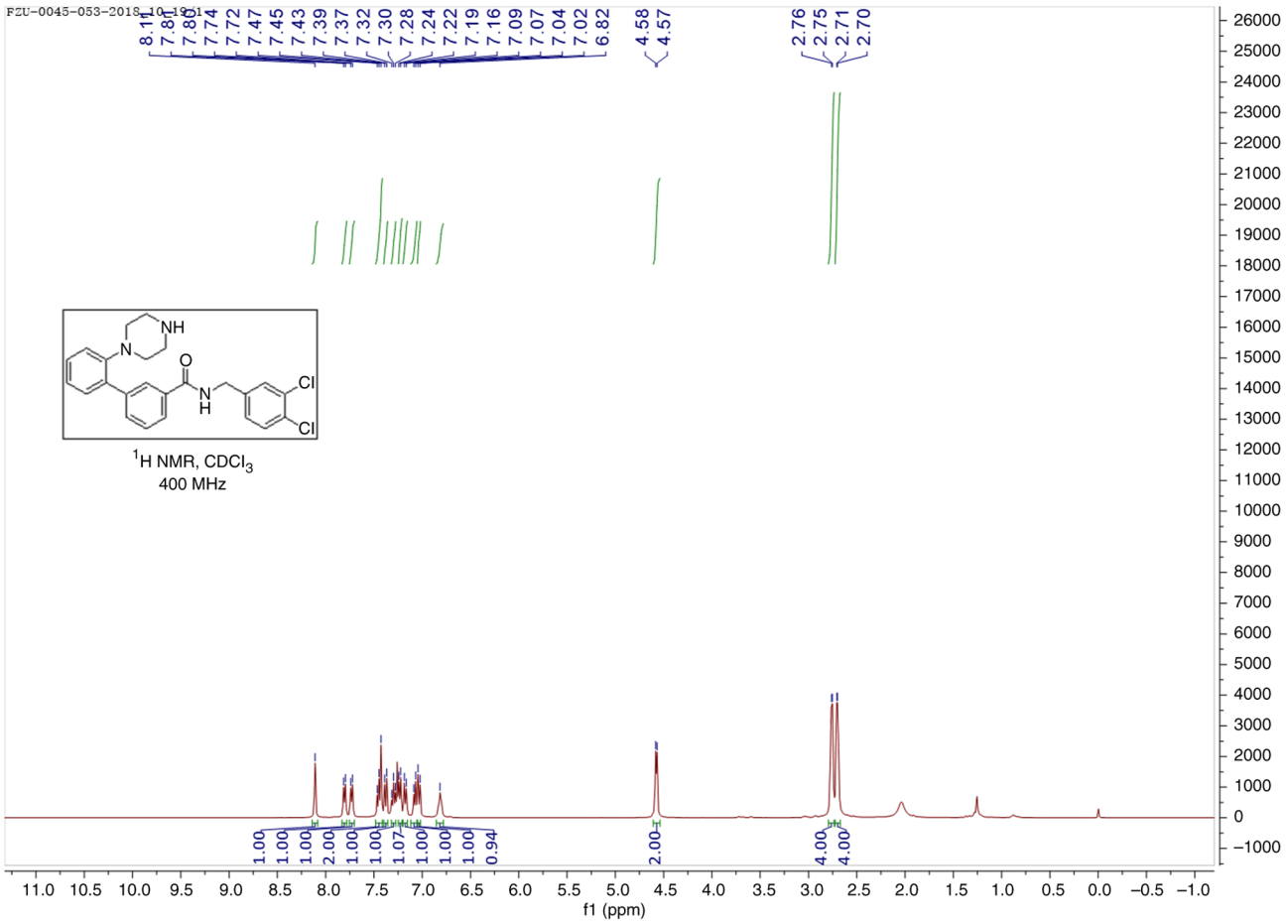


Figure S14. ^{13}C NMR Spectra of compound 053.

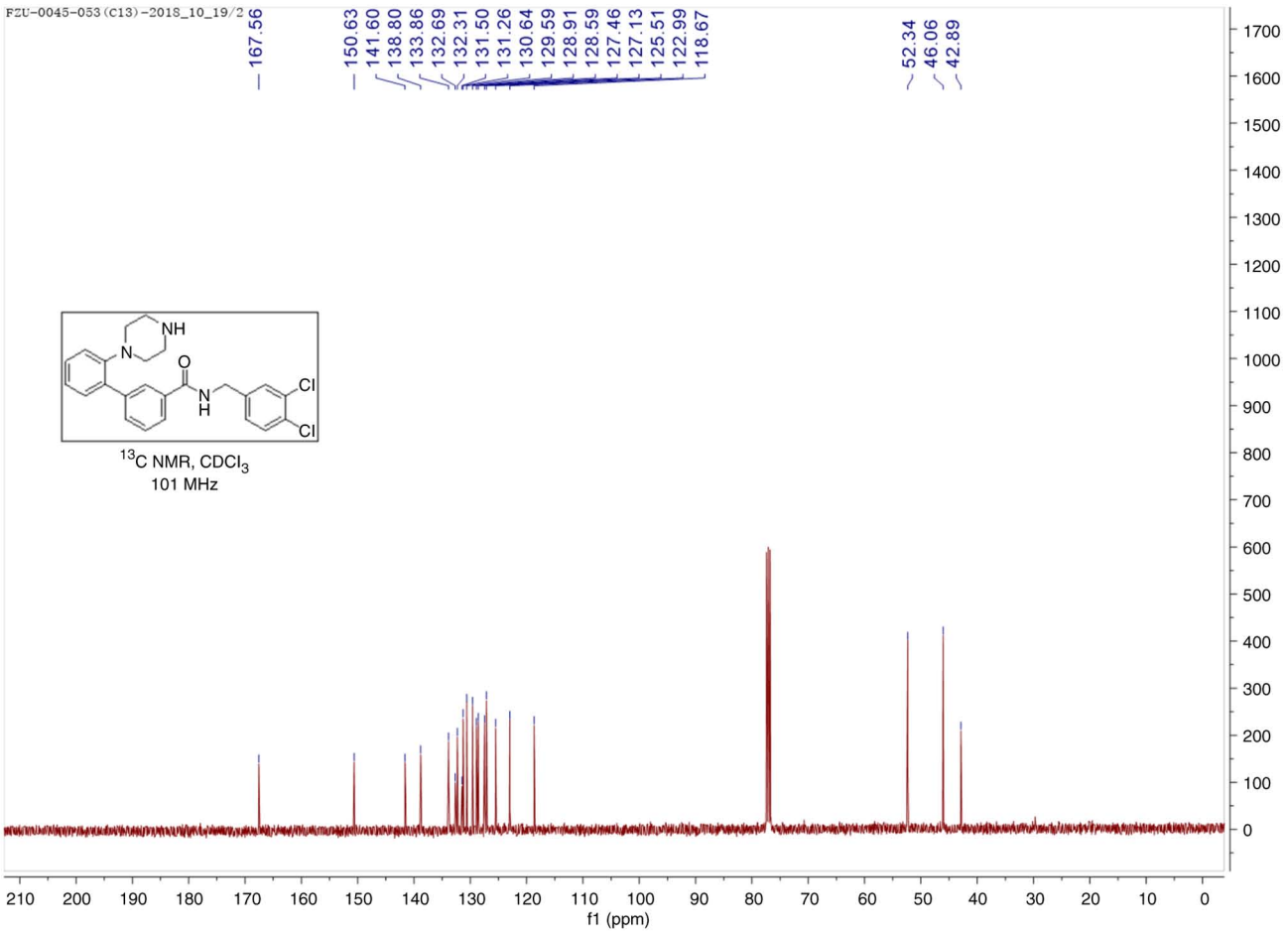


Figure S15. Compound 053 inhibits late autophagy in MCF-7 cells. (A) Representative microscope images of MCF-7 cells treated with different concentrations of 053 (0-20 μM) for 24 h. Scale bar, 40 μm. (B) Internal structure of MCF-7 cells under transmission electron microscopy after treated with 5 μM 053 for 24 h. The lower image is a magnified view of the yellow box in the image above. Scale bar, 1.5 μm. (C) Protein levels of p62 and LC3B-I/II in MCF-7 cells were analyzed by western blotting after treated with 053 (0-10 μM) for 24 h. (D) MCF-7 cells were treated with 5 μM 053 for different times (0, 6, 12, 24 and 48 h), and the protein levels of p62 and LC3B-I/II were analyzed by western blotting. (E) Flow cytometry was used to detect the fluorescence intensity of autophagy-related vesicles stained with CYTO-ID in MCF-7 cells. Cells were treated with 053 (0-5 μM) or CQ (30 μM) for 24 h. The bar graph shows quantitative analysis of relative fluorescence intensity. Data are presented as mean ± SEM (n=3). Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple comparisons test to compare all experimental groups against a single control group. **P<0.01, ****P<0.0001 vs. Control. 053, FZU-0045-053; p62/SQSTM1, sequestosome 1; LC3B, microtubule-associated protein 1A/1B-light chain 3B; CQ, chloroquine.

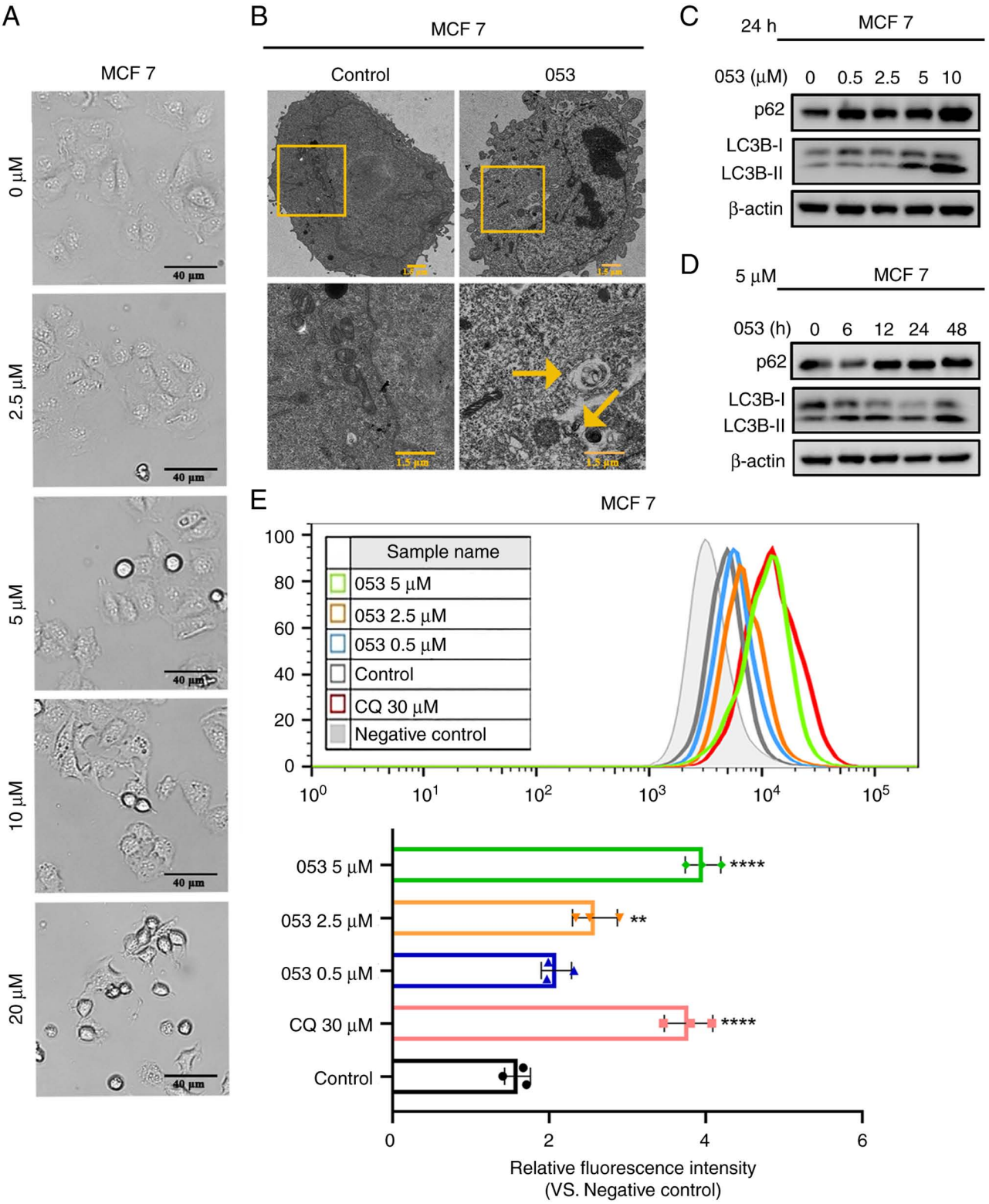


Figure S16. Compound 053 targets autophagic flux in MCF-7 cells. (A) MCF-7 cells were treated with RAPA (10 μM) combined with 053 (5 μM) or CQ (30 μM), or with each drug alone for 24 h. Nuclei were stained with DAPI (blue), and intracellular RFP-LC3 red fluorescence and GFP-LC3 green fluorescence were observed by inverted fluorescence microscope. Scale bar, 20 μm. (B) The number of fluorescence points was analyzed using ImageJ (1.8.0.1) software. Data are presented as mean ± SEM (n=5). Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple comparisons test to compare all experimental groups against a single control group. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs. Control. (C) MCF-7 cells were treated with RAPA (10 μM) in combination with 053 (5 μM) or CQ (30 μM) or the three drugs alone for 24 h. The protein level of LC3B-I/II were analyzed by western blotting. (D) MCF-7 cells were treated with 053 (0-10 μM) for 24 h, the protein levels of AKT, p-AKT, mTOR and p-mTOR were analyzed by western blotting. 053, FZU-0045-053; LC3, microtubule-associated protein 1A/1B-light chain 3; CQ, chloroquine; RAPA, rapamycin.

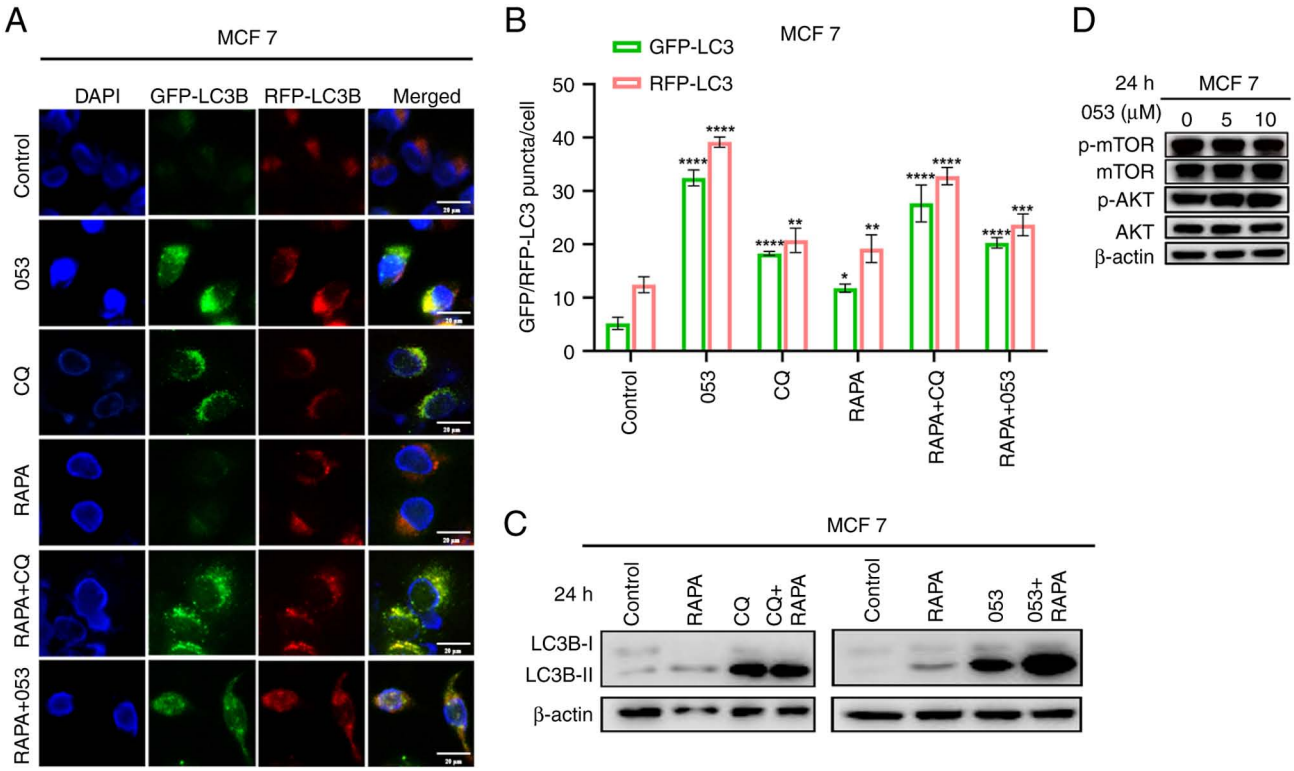
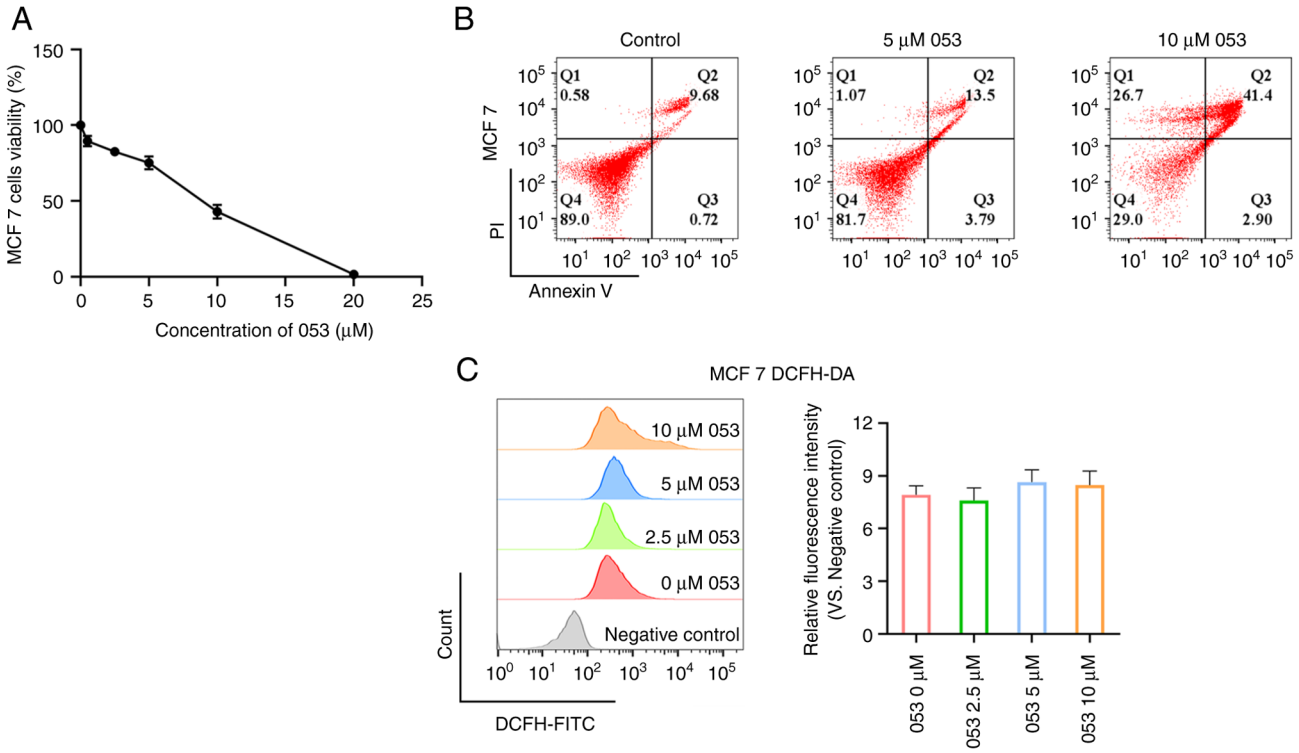



Figure S17. Compound 053 inhibits the proliferation and induces apoptosis in MCF-7 cells. (A) CellTiter 96® AQueous One Solution Cell Proliferation Assay was used to determine the proliferation of MCF-7 cells treated with 053 (0-20 μM) for 24 h. (B) The apoptosis of MCF-7 cells after treated with 053 (0-10 μM) for 24 h was analyzed by flow cytometry. (C) The ROS levels in MCF-7 cells after treatment with 053 (0-10 μM) for 24 h was analyzed by flow cytometry. Data are presented as mean ± SEM (n=3). Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple comparisons test to compare all experimental groups against a single control group. 053, FZU-0045-053; ROS, reactive oxygen species.