Inhibition of Autophagy by a Small Molecule through Covalent Modification of the LC3 Protein

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Abstract: The autophagic ubiquitin-like protein LC3 functions through interactions with LC3-interaction regions (LIRs) of other autophagy proteins including autophagy receptors, which stands out as a promising protein-protein interaction (PPI) target for the intervention of autophagy. Post-translational modifications like acetylation of Lys49 on the LIR-interacting surface could disrupt the interaction, offering an opportunity to design covalent small molecules interfering the interface. Through screening covalent compounds, we discover a small molecule modulator of LC3A/B that covalently modifies LC3A/B protein at Lys49. Activity-based protein profiling (ABPP) based evaluations reveal that a derivative molecule DC-LC3in-D5 exhibits a potent covalent reactivity and selectivity to LC3A/B in HeLa cells. DC-LC3in-D5 compromises LC3B lipidation in vitro and in HeLa cells, leading to deficiency in the formation of autophagic structures and autophagic substrate degradation. DC-LC3in-D5 could serve as a powerful tool for autophagy research as well as for therapeutic interventions.

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1. Chemistry Experiments

Abbreviations and Acronyms

DCM: dichloromethane; EtOAc: ethyl acetate; PE: petroleum ether; ESI: electrospray ionization; h: hour; HPLC: high pressure liquid chromatography; LC: liquid chromatography; LCMS: liquid chromatography mass spectrometry; UPLC: Ultra Performance Liquid Chromatography; M: molar; *m/z*: mass to charge ratio; MeOH: methanol; min: minutes; MS: mass spectrometry; $NaOC_2H_5$: sodium ethoxide; $NH_3{\cdot}H_2O$: ammonium hydroxide; NMR: nuclear magnetic resonance; RT: room temperature; THF: Tetrahydrofuran; TLC: thin—layer chromatography; v/v: volume per unit volume;

General Experimental Conditions

Commercially available chemicals were used without further purification. All products were characterized by their NMR and MS spectra. ^{1}H NMR spectra were recorded with a Bruker AM-300 and a Varian-MERCURY Plus-400 NMR spectrometer.^{13}C NMR spectra were recorded with a Varian-MERCURY Plus-500 NMR spectrometer. Chemical shifts are reported in ppm (δ scale) as referenced to TMS and coupling constant (J) values are reported in hertz (Hz). Data are presented as follows: chemical shift, multiplicity (s = singlet, d = doublet, dd = doublet of doublet, t = triplet, q = quartet, m = multiplet, br = broad), coupling constant in Hz, and integration. High-resolution mass spectrometry (HRMS) were recorded on a Thermo – DFS spectrometer. Column chromatography was performed using silica gel (200–300 mesh). Analytical TLC was performed silica gel plates and visualized under ultraviolet light (254 nm).

Liquid Chromatography Mass Spectrometry (LCMS)

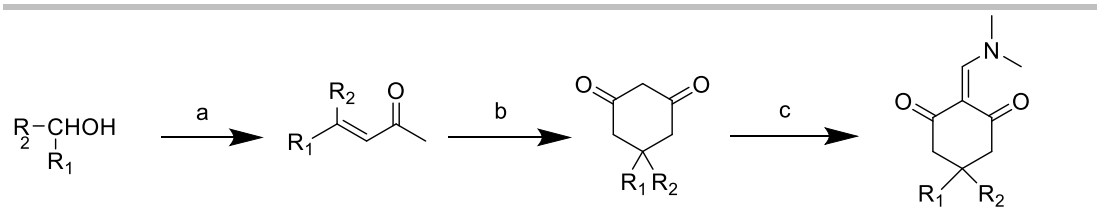
Liquid Chromatography Mass Spectrometry (LCMS) experiments to determine retention times (R_T) and associated mass ions were performed using the following methods:

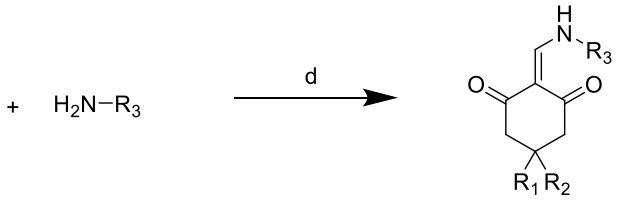
The system consisted of a Waters SQD2 quadrupole mass spectrometer linked to a Waters UPLC H-class Infinity LC system with UV diode array detector and autosampler. The spectrometer consisted of an electrospray ionization source operating in positive and negative ion mode. LCMS experiments were performed on each sample submitted using the following conditions: LC Column: ACQUITY UPLC®BEH C18 RRHD, 1.7 µm, 50 x 2.1 mm maintained at 40 °C. Mobile phases: A) 0.1% (v/v) formic acid in water; B) acetonitrile.

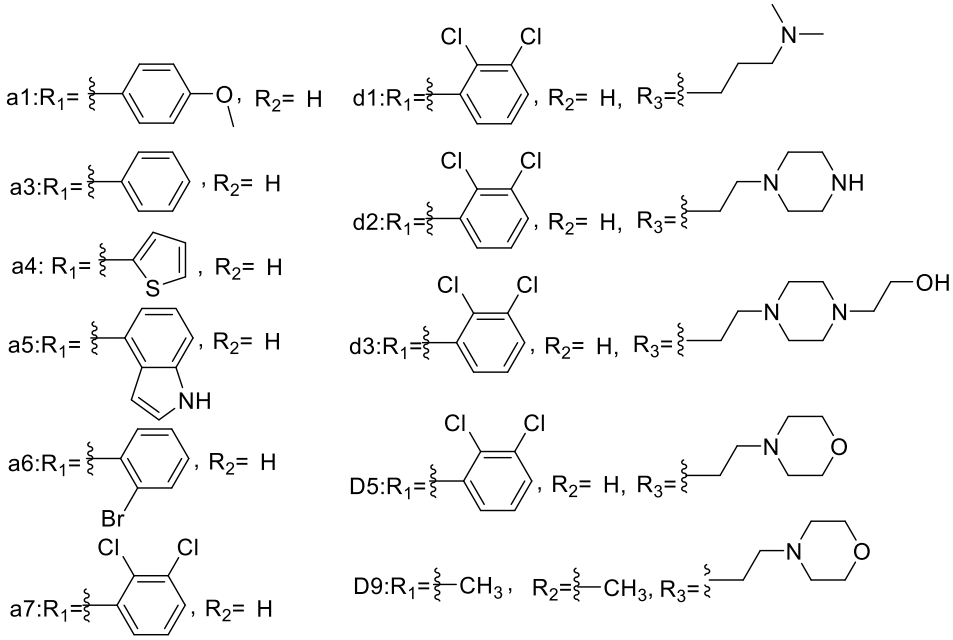
Retention Time (min, t_R)	Flow (mL/min)	%A	%B
0.00	0.5	90	10
4.00	0.5	0	100
4.50	0.5	0	100
4.51	0.5	90	10
5.00	0.5	90	10

1. Procedure A (step 1 to step 4) for synthesis of compounds a1, a3, a4, a5, a6, a7, d1, d2, d3, DC-LCin-D5, DC-LCin-D9. Synthesis of the presented compounds were performed according to Schemes 1. Synthesis and analytical data for representative compounds are provided below.

Scheme 1. Synthesis of Compound a1, a2, a3, a4, a5, a6, a7, DC-LC3in-D5, DC-LC3in-D9.







(a). $Ph_3P=CHCOCH_3$, PhMe,125 ^{0}C,2h; (b). (i). ethyl malonate, $NaOC_2H_5$, C_2H_5OH, 90 ^{0}C, 3h; (ii). NaOH, H_2O, 100 ^{0}C, 2h; (iii). H_3O+, 100 ^{o}C, 2h; (c). 1,1-dimethoxy-N,N-dimethylmethanamine, CH_2Cl_2, rt, 1h; (d). CH_2Cl_2, rt, 12h.

Step1:

To a solution of Aldehyde 1 (20 mmol, 1.0 equiv) in toluene (30 mL) was added 1-(triphenylphosphoranylidene)-2-propanone (22 mmol, 1.1 equiv) at room temperature. The reaction mixture was refluxed for 2 h. After completion, the reaction mixture was extracted with EtOAc and H_2O. The combined organic layers were washed with brine, water, and dried over Na_2SO_4 and then concentrated under vacuum. The residue was purified by column chromatography with petroleum ether/ethyl acetate (20/1-10/1, v/v) to obtain compound **2** (86-98% yield). [1]

Step2:

To a solution of 21% sodium ethoxide (10 mmol, 1.0 equiv) in ethanol (20 mL), diethyl malonate (1.75 mL, 11mmol, 1.0 equiv) was added dropwise at 0 ºC, and then reaction mixture was stirred at room temperature for 0.5 h. Then, intermediate 2 (10 mmol, 1.0 equiv) in ethanol (10mL) was added dropwise and the mixture was stirred at reflux and monitored by LC-MS until the corresponding starting material was consumed. After cooling, ethanol was removed under vacuum. The residue was treated with 5M sodium hydroxide (20 mmol, 2.0 equiv) and heated at 100 ºC for 2h. Then the reaction mixture was treated with 6M HCl until Ph = 2 at 0 ºC, refluxed for 2 h and left to cool at room temperature. The solid thus formed was isolated by filtration to yield intermediate **3**(43-72% yield), which was used directly in the next step. [2]

Step3:

Compound **3** (5 mmol, 1.0 equiv) was dissolved in DCM (10 mL), and 1,1-dimethoxy-N,N-dimethylmethanamine (15 mmol, 3.0 equiv) was added. The mixture was allowed to stir at room temperature for 1 h. Solvent was removed under vacuum, and the residue was purified by column chromatography with DCM/MeOH (100/1-50/1, v/v) to provide compound **4, a1, a3, a4, a5, a6,a7** (75-98% yield).

Step4:

Intermediate **4** (2 mmol, 1.0 equiv) and amine (2.4 mmol, 1.2 equiv) **5** was dissolved in DCM (10 mL). The mixture was allowed to stir at room temperature overnight. After completion, the solution was removed in vacuo, the residue was purified by column chromatography (DCM/MeOH = 200/1-50/1) to give final product **d1, d2, d3, D5, D9** (73-97% yield). [3]

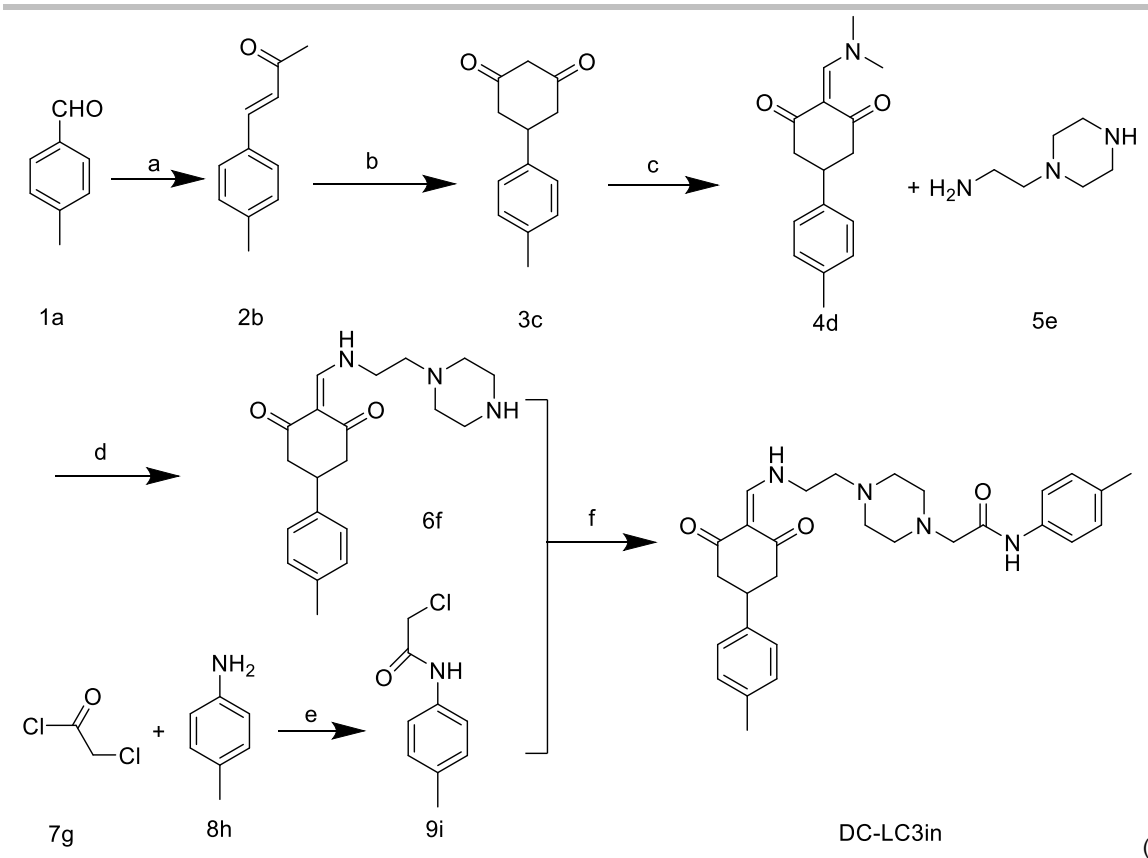
2-((dimethylamino)methylene)-5-(4-methoxyphenyl)cyclohexane-1,3-dione (a1). LCMS : RT = 2.42 min (purity >98% at 254 nm), *m/z* = 273 $[M+H]^+$. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.04 (d, *J* = 16.8 Hz, 1H), 7.16 (d, *J* = 8.4 Hz, 2H), 6.86 (d, *J* = 8.6 Hz, 2H), 3.79 (s, 3H), 3.41 (s, 3H), 3.30 (td, *J* = 11.1, 5.4 Hz, 1H), 3.19 (d, *J* = 11.1 Hz, 3H), 2.69 (qd, *J* = 16.8, 8.1 Hz, 4H).^{13}C NMR (126 MHz, $CDCl_3$) δ 195.1, 162.4, 140.2, 136.3, 129.4, 126.5, 108.8, 48.6, 45.7, 44.8, 36.9, 21.0. HRMS (ESI, positive) m/z calcd for $C_{16}H_{19}NO_3$ $[M + H]^+$: 273.1403. Found: 273.1423.
2-((dimethylamino)methylene)-5-phenylcyclohexane-1,3-dione (a3). LCMS : RT = 0.35 min (purity >99% at 254 nm), *m/z* = 244 $[M+H]^+$. 1H NMR (300 MHz, CDCl3) δ 8.07 (s, 1H), 7.37 – 7.29 (m, 2H), 7.24 (d, J = 7.2 Hz, 3H), 3.41 (s, 3H), 3.33 (dd, J = 10.8, 5.5 Hz, 1H), 3.22 (s, 2H), 2.81 – 2.60 (m, 4H). 13C NMR (126 MHz, CDCl3) δ 194.61, 161.99, 142.84, 128.35, 126.49, 126.37, 126.28, 108.35, 48.27, 45.14, 44.42, 36.93. HRMS (ESI, positive) m/z calcd for $C_{15}H_{17}NO_2$ $[M + H]^+$: 244.1346. Found: 244.1361.
2-((dimethylamino)methylene)-5-(thiophen-2-yl)cyclohexane-1,3-dione(a4). LCMS : RT = 2.76 min (purity >99% at 254 nm), *m/z* = 250 $[M+H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08 (s, 1H), 7.19 (d, *J* = 5.2 Hz, 1H), 6.99 – 6.92 (m, 1H), 6.88 (d, *J* = 3.2 Hz, 1H), 3.71 – 3.59 (m, 1H), 3.43 (s, 3H), 3.22 (s, 3H), 2.92 (dd, *J* = 16.8, 4.0 Hz, 2H), 2.74 (dd, *J* = 16.8, 10.8 Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 194.0, 162.4, 147.3, 126.8, 123.5, 123.1, 108.8, 48.6, 46.0, 44.8, 32.9. HRMS (ESI, positive) m/z calcd for $C_{13}H_{15}NO_2S$ $[M + H]^+$: 250.0843. Found: 250.0898.
2-((dimethylamino)methylene)-5-(1H-indol-4-yl)cyclohexane-1,3-dione(a5) LCMS : RT = 0.85 min (purity >99% at 254 nm), *m/z* = 283 $[M+H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.35 (s, 1H), 8.14 (s, 1H), 7.33 (d, J = 8.2 Hz, 1H), 7.26 – 7.23 (m, 1H), 7.22 – 7.17 (m, 1H), 7.00 (d, J = 7.3 Hz, 1H), 6.63 (ddd, J = 3.1, 2.0, 0.9 Hz, 1H), 3.84 (ddd, J = 16.6, 10.8, 5.8 Hz, 1H), 3.44 (s, 3H), 3.27 (s, 3H), 2.95 – 2.85 (m, 4H). 13C NMR (126 MHz, CDCl3) δ 195.32, 162.05, 135.54, 134.79, 126.03, 123.73, 121.77, 115.59, 109.49, 108.46, 100.12, 76.94, 76.69, 76.44, 48.22, 44.45, 44.40, 34.61. HRMS (ESI, positive) m/z calcd for $C_{17}H_{18}N_2O_2$ $[M + H]^+$: 283.1412. Found: 283.1425.

5-(2-bromophenyl)-2-((dimethylamino)methylene)cyclohexane-1,3-dione(a6)
LCMS : RT = 0.56 min (purity >98% at 254 nm), *m/z* = 322 $[M+H]^+$. 1H NMR (400 MHz, CDCl3) δ 8.12 (s, 1H), 7.60 (d, J = 7.7 Hz, 1H), 7.34 (t, J = 7.6 Hz, 1H), 7.27 (s, 1H), 7.14 (dd, J = 10.6, 4.6 Hz, 1H), 3.83 (ddd, J = 15.8, 7.9, 4.0 Hz, 1H), 3.45 (s, 3H), 3.27 (s, 3H), 2.81 (dd, J = 16.8, 4.1 Hz, 2H), 2.65 (dd, J = 16.7, 12.1 Hz, 2H). 13C NMR (126 MHz, CDCl3) δ 194.18, 161.97, 141.49, 132.92, 127.86, 127.54, 126.63, 124.05, 108.25, 76.92, 76.66, 76.41, 48.31, 44.49, 43.77, 35.99. HRMS (ESI, positive) m/z calcd for $C_{15}H_{16}BrNO_2$ $[M + H]^+$: 322.0432. Found: 322.0446.
5-(2,3-dichlorophenyl)-2-((dimethylamino)methylene)cyclohexane-1,3-dione (a7). LCMS : RT = 0.42 min (purity >99% at 254 nm), *m/z* = 312 $[M+H]^+$. 1H NMR (500 MHz, CDCl3) δ 8.10 (s, 1H), 7.38 (dd, J = 7.6, 2.0 Hz, 1H), 7.22 (t, J = 7.7 Hz, 1H), 7.18 (dd, J = 7.9, 1.9 Hz, 1H), 3.89 (tt, J = 11.9, 4.0 Hz, 1H), 3.44 (s, 3H), 3.25 (s, 3H), 2.78 (dd, J = 16.8, 4.1 Hz, 2H), 2.62 (dd, J = 16.8, 11.9 Hz, 2H). 13C NMR (126 MHz, CDCl3) δ 193.78, 161.99, 142.33, 133.24, 131.62, 128.43, 127.20, 124.63, 108.17, 76.92, 76.66, 76.41, 48.34, 44.49, 43.49, 34.27. HRMS (ESI, positive) m/z calcd for $C_{15}H_1Cl_2NO_2$ $[M + H]^+$: 312.0549. Found: 312.0568.
5-(2,3-dichlorophenyl)-2-(((2-morpholinoethyl)amino)methylene) cyclohexane-1,3-dione(DC-LC3in-D5) LCMS : RT = 1.82 min (purity >99% at 254 nm), *m/z* = 398, 401, 402 $[M+H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.21 (s, 1H), 8.20 (d, *J* = 14.4 Hz, 1H), 7.38 (dd, *J* = 7.6, 1.6 Hz, 1H), 7.23 – 7.14 (m, 2H), 3.89 (tt, *J* = 11.8, 4.0 Hz, 1H), 3.77 – 3.70 (m, 4H), 3.53 (dd, *J* = 12.0, 6.0 Hz, 2H), 2.78 (dddd, *J* = 16.8, 6.0, 4.0, 2.0 Hz, 2H), 2.70 – 2.57 (m, 4H), 2.53 – 2.47 (m, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 197.6, 194.8, 158.9, 142.5, 133.7, 132.0, 128.9, 127.6, 125.0, 108.0, 66.9, 57.5, 53.5, 47.0, 43.4, 43.0, 35.0. HRMS (ESI, positive) m/z calcd for $C_{19}H_{22}Cl_2N_2O_3$ $[M + H]^+$: 398.1103. Found: 398.1111.

5,5-dimethyl-2-(((2-morpholinoethyl)amino)methylene)cyclohexane-1,3-dione(DC-LC3in-D9) LCMS : RT = 0.34 min (purity >98% at 254 nm), *m/z* = 281 $[M+H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.12 (s, 1H), 8.13 (d, *J* = 14.4 Hz, 1H), 3.78 – 3.69 (m, 4H), 3.50 (q, *J* = 6.0 Hz, 2H), 2.60 (t, *J* = 6.0 Hz, 2H), 2.53 – 2.46 (m, 4H), 2.36 (d, *J* = 14.3 Hz, 4H), 1.06 (s, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 199.0, 196.3, 158.3, 107.6, 66.9, 57.6, 53.5, 51.5, 51.1, 46.9, 31.2, 28.6. HRMS (ESI, positive) m/z calcd for $C_{15}H_{24}N_2O_3$ $[M + H]^+$: 281.1809. Found: 281.1856.

2. Procedure B of the synthesis of 2-(4-(2-(((2,6-dioxo-4-(p-tolyl) cyclohexylidene)methyl)amino)ethyl)piperazin-1-yl)-N-(p-tolyl) acetamide (DC-LC3in).

Scheme 2. Synthesis of Compound DC-LC3in**.**



(a). $Ph_3P=CHCOCH_3$, PhMe,125 ^{0}C,2h; (b). (i). ethyl malonate, $NaOC_2H_5$, C_2H_5OH, 90 ^{0}C, 3h; (ii). NaOH, H_2O, 100 ^{0}C, 2h; (iii). H_3O+, 100 ^{o}C, 2h; (c). 1,1-dimethoxy-N,N-dimethylmethanamine, CH_2Cl_2, rt, 1h; (d). CH_2Cl_2, rt, 12h; (e). Et_3N, CH_2Cl_2, 0 ^{0}C - rt, 3h; (f). K_2CO_3, KI, CH_3CN, 85 ^{0}C, 3h.

Compounds **2b** to **6f** were prepared according to procedure A, step 1 to step 4.

Step 5:

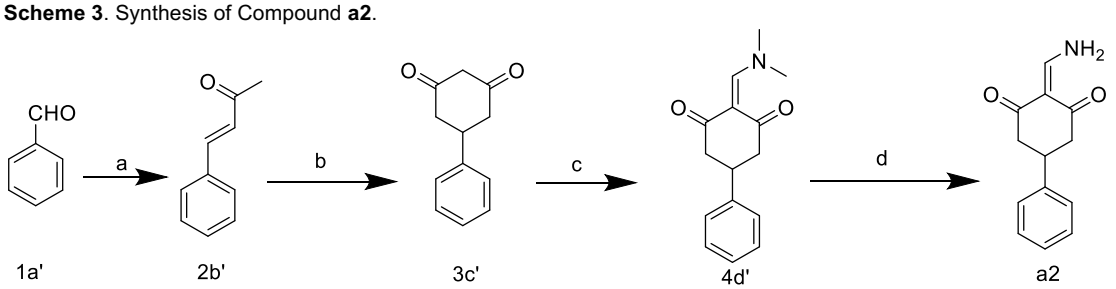
To a solution of p-toluidine **8h**(536mg, 5 mmol, 1.0 equiv) in DCM were added 2-chloroacetyl chloride **7g**(678mg, 6 mmol, 1.2 equiv), and triethylamine (1.63Ml, 10 mmol, 2.0 equiv) at 0 °C for 0.5 h. After being stirred at room temperature for 2.5 h, the reaction was quenched with saturated $NaHCO_3$ solution and extracted with DCM. The combined organic layers were washed with brine, and dried over Na_2SO_4, and concentrated. The residue was purified by column chromatography with petroleum ether/ethyl acetate (50/1-10/1, v/v) to obtain compound **9i** (860mg, 94% yield).[4,5]

Step 6:

Compound **6f** (684mg, 2.0 mmol, 1.0 equiv) and **9i** (406 mg, 2.2 mmol, 1.1 equiv) were dissolved in CH_3CN (15 mL), and K_2CO_3(332mg, 2.4 mmol, 1.2 equiv), KI (80mg, 0.4 mmol, 0.2 equiv) were added, and the mixture was stirred at reflux for 3 h. The mixture was diluted with H_2O, and extracted with DCM. The organic layers were combined, washed with brine, dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (DCM/MeOH =50/1, v/v) to give compound **DC-LC3in** (878mg, 90% yield).[4,5] [1] LCMS : RT = 2.30 min (purity >99% at 254 nm), *m/z* = 490, 491[M+H]$^+$. H NMR (400 MHz, $CDCl_3$) δ 11.29 – 11.10 (m, 1H), 9.02 (s, 1H), 8.18 (d, *J* = 14.5 Hz, 1H), 7.45 (d, *J* = 8.3 Hz, 2H), 7.14 (d, *J* = 2.6 Hz, 6H), 3.52 (d, *J* = 5.9 Hz, 2H), 3.31 (s, 1H), 3.15 (s, 2H), 2.79 – 2.54 (m, 14H), 2.33 (d, *J* = 4.5 Hz, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 199.3, 196.5, 168.8, 159.5, 140.8, 137.2, 135.8, 134.5, 130.2, 130.2, 127.2, 120.2, 108.9, 62.5, 57.7, 54.1, 53.9, 47.9, 45.9, 45.5, 37.9, 21.7, 21.6. HRMS (ESI, positive) m/z calcd for $C_{29}H_{36}N_4O_3$ [M + H]$^+$: 490.2852. Found: 490.2891.

3. Procedure C of the synthesis of 2-(aminomethylene)-5-phenylcyclohexane-1,3-dione(a2)

Scheme 3. Synthesis of Compound **a2**.



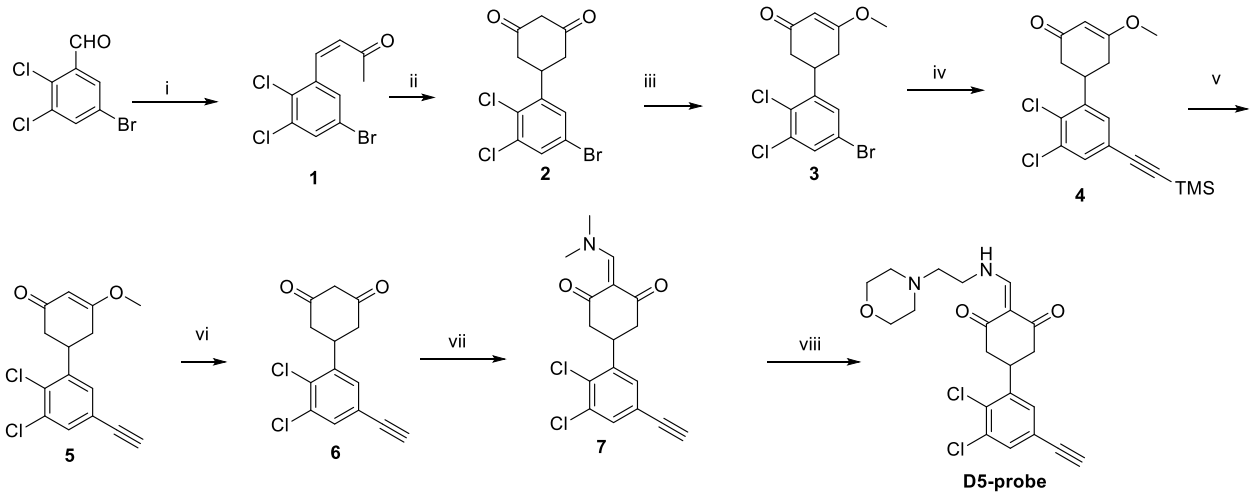
(a). $Ph_3P=CHCOCH_3$, PhMe,125 ^{0}C,2h; (b). (i). ethyl malonate, $NaOC_2H_5$, C_2H_5OH, 90 ^{0}C, 3h; (ii). NaOH, H_2O, 100 ^{0}C, 2h; (iii). H_3O+, 100 ^{o}C, 2h; (c). 1,1-dimethoxy-N,N-dimethylmethanamine, CH_2Cl_2, rt, 1h; (d). $NH_3{\cdot}H_2O$, THF, 12h, RT.

Compounds **2b'** to **4d'** were prepared according to procedure A, step 1 to step 3.

Step 5: Intermediate **4d'** (972 mg, 4 mmol, 1.0 equiv) and $NH_3{\cdot}H_2O$ (0.18 ml, 4.4 mmol, 1.1 equiv) was dissolved in THF (10 mL). The mixture was allowed to stir at room temperature overnight. After completion, the solution was removed in vacuo. The residue was purified by column chromatography (DCM/MeOH = 100/1-25/1) to give final product **a2**(93% yield).[2] LCMS: RT = 0.42 min (purity >97% at 254 nm), *m/z* = 216 $[M+H]^+$. ^1H NMR (300 MHz, $CDCl_3$) δ 10.53 (s, 1H), 8.27 (dd, *J* = 15.6, 9.0 Hz, 1H), 7.35 (t, *J* = 7.5 Hz, 2H), 7.23 (d, *J* = 7.8 Hz, 3H), 6.69 (s, 1H), 3.36 (s, 1H), 2.83 – 2.60 (m, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 199.5, 196.3, 158.3, 142.8, 128.9, 127.0, 126.6, 109.2, 45.4, 44.8, 37.4. HRMS (ESI, positive) m/z calcd for $C_{13}H_{13}NO_2$ $[M + H]^+$: 216.1003. Found: 216.1019.

4. Procedure D of the synthesis of 5-(2,3-dichloro-5-ethynylphenyl)-2-(((2-morpholinoethyl) amino) methylene) cyclohexane-1,3-dione (D5-probe).

Scheme 4. Synthesis of Compound **D5-probe**.



Reagents and conditions: (i) Ph3P=CHCOCH3, PhMe, 125℃, 2h; (ii) (a) Ethyl malonate, NaOC2H5, C2H5OH, 90 ℃, 3h; (b) NaOH, H2O, 100 ℃, 2h; (c) 6M HCl, 100 ℃, 2h; (iii) H2SO4, MeOH, 65℃, 12h. (iv) Trimethylsilylacetylene, CuI, Pd(Ph3P)2Cl2, EtOH, TEA, rt, 4h; (v) TBAF, THF, rt, 4h; (vi) 1M HCl, THF, rt, 6h; (vii) 1,1-dimethoxy-N,N-dimethylmethanamine, DCM, rt, 1h; (viii) 4-(2-aminoethyl) morpholine, DCM, rt, 12h.

Step 1:

To a solution of 5-bromo-2,3-dichlorobenzaldehyde (254mg, 1mmol) in toluene (7 mL) was added 1-(triphenylphosphoranylidene)-2-propanone (477mg, 1.5mmol) at room temperature. The reaction mixture was stirred at 125℃ for 2 h. After completion, the reaction mixture was extracted with EtOAc and H2O. The combined organic layers were washed with brine, water, and dried over Na2SO4 and then concentrated under vacuum. The residue was purified by column chromatography with petroleum ether/ethyl acetate (20/1-10/1, v/v) to obtain intermediate 1 (198mg, 67%).

Step 2:

To a solution of 20% sodium ethoxide in ethanol (550uL, 1.63mmol), diethyl malonate (247uL, 1.63mmol) was added dropwise at 0 ℃, and then reaction mixture was stirred at room temperature for 0.5 h. Then, intermediate 1 (320mg, 1.08mmol) in ethanol (5mL) was added dropwise and the mixture was stirred at reflux and monitored by LC-MS until the corresponding starting material was consumed. After cooling, ethanol was removed under vacuum. The residue was treated with 5M sodium hydroxide (86mg, 2.16mmol) and heated at 100 ℃ for 2h. Then the reaction mixture was treated with 6M HCl until pH = 2, then refluxed for 2 h and left to cool at room temperature. The solid thus formed was isolated by filtration to yield intermediate 2(336mg, 93%), which was used directly in the next step.

Step 3:

To a solution of intermediate 2 (303mg, 0.907mmol) in MeOH (10mL), H_2SO_4 (1.5mL) was added dropwise at 0 ℃, and then stirred at 65 ℃ for 12h. The reaction mixture was treated with saturated NaHCO3 solution until pH = 7, then extracted with EtOAc. The combined organic layers were washed with brine, water, and dried over Na_2SO_4 and then concentrated under vacuum. The residue was purified by column chromatography with petroleum ether/ethyl acetate (15/1-10/1, v/v) to obtain intermediate 3 (170mg, 53.5%).

Step 4:

To a solution of intermediate 3 (170mg, 0.485mmol), trimethylsilylacetylene (137uL, 0.97mmol) in the mixture of EtOH (5mL) and TEA (1.25mL) was added palladium bis(triphenylphosphine) dichloride (68mg, 0.097mmol) and CuI (18mg, 0.097mmol) under the protection of argon. The reaction was stirred at room temperature for 4h. After cooling, the mixture was extracted with ethyl acetate (5ml × 3) and the combine organic layers were dried over anhydrous sodium sulfate then concentrated in vacuum. The residue was purified by column chromatography with petroleum ether/ethyl acetate (15/1-10/1, v/v) to give intermediate 4 (137mg, 77%).

Step 5:

To a solution of intermediate 4 (20mg, 0.059mmol) in THF (3mL), 1M tetrabutylammonium fluoride (89uL, 0,089mmol) in THF was added. The reaction was stirred at room temperature for 4h. The mixture was extracted with ethyl acetate (3ml × 3) and the combine organic layers were dried over anhydrous sodium sulfate then concentrated in vacuum without further purification to give intermediate **5** (12mg, 70%).

Step 6:

To a solution of intermediate **5** (12mg, 0.040mmol) in THF (1mL), 1M HCl (0.1mL) was added. The reaction was stirred at room temperature for 6h. The solution was removed in vacuo, the residue was purified by column chromatography (with petroleum ether/ethyl acetate (10/1-5/1, v/v) to give intermediate **6** (10mg, 90%).

Step 7:

Intermediate **6** (10mg, 0.035mmol), and 1,1-dimethoxy-N,N-dimethylmethanamine (9.2 uL, 0.070mmol) was dissolved in DCM (2 mL). The mixture was stirred at room temperature for 1h. The solution was removed in vacuo, the residue was purified by column chromatography (DCM/MeOH = 100/1-50/1) to give intermediate **7** (9mg, 76%).

Step 8:

Intermediate **7** (9mg, 0.026mmol) was dissolved in DCM, then 4-(2-aminoethyl) morpholine (3.4 uL, 0.026mmol) was added. The mixture was stirred at room temperature for 12h. The solvent was removed under vacuum, the residue was purified by column chromatography with DCM/MeOH (100/1-50/1, v/v) to provide compound **D5-probe** (8mg, 75%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.27 – 11.16 (m, 1H), 8.19 (d, *J* = 14.4 Hz, 1H), 7.50 (d, *J* = 1.7 Hz, 1H), 7.26 (d, *J* = 1.7 Hz, 1H), 3.89 – 3.80 (m, 1H), 3.77 – 3.71 (m, 4H), 3.57 – 3.50 (m, 2H), 3.15 (s, 1H), 2.79 – 2.71 (m, 2H), 2.65 – 2.58 (m, 4H), 2.54 – 2.48 (m, 4H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 197.36, 194.55, 159.10, 142.67, 133.86, 132.97, 132.18, 128.73, 122.02, 108.01, 81.47, 79.50, 67.00, 57.59, 53.58, 47.09, 43.31, 42.93, 35.06. HRMS (ESI, positive) m/z calcd for $C_{21}H_{23}Cl_2N2O_3$ $[M + H]^+$: 421.1080. Found: 421.1071.

2. Cell culture and Stable Cell Line Generation

HeLa cells and HEK293T cells were cultured in Dulebcco's Modified Eagle's Medium (DMEM, Invitrogen) with 10% fetal bovine serum, 1% penicillin and streptomycin (Invitrogen). To generate stable cell lines expressing EGFP-LC3B, lentivirus containing EGFP-LC3B was used to infect HeLa cells. To generate stable cell lines expressing TurboRFP-EGFP-GABARAP, we constructed lentivirus overexpression plasmid based on CMV-TurboRFP-EGFP-LC3-PGK-puro from Genomeditech (GM-1314L204H). Then HeLa cells were infected with this lentivirus. Stable cell clones were obtained by screening of puromycin (Invitrogen).

3. Cloning

cDNA encoding human LC3B (NCBI accession number NP_073729.1) was bought from Addgene. cDNA encoding human LC3A, LC3C, GABARAP, GABARAPL1 and GABARAPL2 (NCBI accession number NP_115903.1, NP_001004343.1, NP_009209.1, NP_113600.1, NP_009216.1) was obtained from synthesis. The cDNAs were used as templates for the amplification of the LC3B and its homologues in the polymerase chain reaction (PCR). PCR products were purified and cloned into a pGEX-6P-1, pGEX-4T-1 or pcDNA3.1 expression vector.

For overexpressing ATG7 or ATG3 in cells, human Atg7 (NM_001349234.2) or Atg3 (NM_022488.5) sequence was obtained from synthesis and were inserted in pCMV vector with N-terminal FLAG (ATG7) or C-terminal FLAG (ATG3).

4. Protein Expression and Purification

For the FP (Fluorescence Polarization), GST-LC3B (1-125) and its mutants or GST-GABARAP were expressed in E. coli BL21 (DE3) cells in LB (Luria-Bertani) medium by inducing with 0.4 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) for 18 hrs at 16°C. The cells were harvested by centrifugation (5000 rpm for 10 min at 4°C, JLA 81,000 rotor, on a Beckman Coulter Avanti J-20 XP centrifuge) and re-suspended in 20 mM Tris-HCl pH7.5 buffer with 150 mM NaCl and 5 mM DTT (Dithiothreitol). The cell debris was removed by centrifugation (18000 rpm for 35 min at 4°C, JA 20.1 rotor, on a Beckman Coulter Avanti J-20 XP centrifuge) after sonication. The supernatant was applied to an immobilized glutathione column (GSTrap FF, GE Healthcare Life Sciences) and eluted with elution buffer (50 mM Tris-HCl pH8.5, 150 mM NaCl, 5 mM DTT, 10mM GSH).The protein was further purified with a Superdex 200 10/300 GL (GE Healthcare Life Sciences) gel filtration column on an ÄKTA pure system (GE Healthcare Life Sciences). Proteins were concentrated to 5-10 mg/mL in gel filtration buffer (50 mM HEPES pH7.5, 150 mM NaCl, 5 mM DTT). For -80°C storage, additional 5% glycerol was added to the sample.For ITC (isothermal titration calorimetry), Protein stability shift assay and molecular level Mass spectrometry studies, GST-LC3B (1-125) and its mutants are expressed and purified as above procedures. The GST fusion proteins were treated with PPase overnight at 4°C to remove the GST tag. The following purifications were performed with a Superdex 75 10/300 GL or HiLoad 16/600 Superdex 75 pg (GE Healthcare Life Sciences) gel filtration column on an ÄKTA pure system (GE Healthcare Life Sciences). Proteins were concentrated to 1-5 mg/mL in gel filtration buffer (50 mM HEPES pH7.5, 150 mM NaCl, 1 mM DTT).

For NMR titration assays, 15N-labeled LC3B (1-120) was expressed in E. coli BL21 (DE3) cells in the medium containing 15NH4Cl by inducing with 0.6 mM IPTG for 18 hrs at 16°C. Cells were harvested by centrifugation and the sonication was conducted with buffer (25 mM sodium phosphate (pH7.5), 100 mM NaCl, 5 mM DTT). The supernatant was applied to an immobilized glutathione column and eluted with elution buffer (25 mM sodium phosphate pH7.5, 100 mM NaCl, 5mM DTT, 10mM GSH). The GST tag was removed by PPase overnight at 4°C and further purification was performed with a Superdex 75 10/300 GL gel filtration column on an ÄKTA pure system. Proteins were concentrated to 1-5 mg/mL in gel filtration buffer (25 mM sodium phosphate pH7.0, 100 mM NaCl).

For X-ray crystallography, LC3B (2-119) was expressed as above. Proteins were concentrated to 20 mg/mL in gel filtration buffer (50 mM HEPES pH7.5, 1 mM DTT).

For LC3-lipidation assays and ATG7 conjugation assays, ATG7, ATG3, ATG12–ATG5-ATG16N (11-43), LC3B, and GABARAP proteins were prepared as described previously[6].

5. Fluorescence Polarization (FP) Assays for Inhibition of LC3-LIR Interaction

N-terminal FITC-labeled LBP2 peptide (FITC-LBP2, sequence: FITC-GGDDDWTHLSSKEVD) was used at the final concentration of 18 nM and recombinant GST-LC3B at 180 nM in the FP Buffer (50 mM HEPES pH 7.5; 1 mM DTT; 0.1 mg/mL BSA). Compounds were added and incubated in the dark at 25°C before adding FITC-labeled peptide.Changes in fluorescence polarization were monitored at 535 nm after excitation at 480 nm using the PerkinElmer Envison protocol and applied to determine the inhibition ratio and IC_{50} values with the GraphPad Prism 8.0 program. All the IC_{50} values were determined at the incubation time of 18 hrs. For the determination of K_{inact}/K_i, different concentrations of compounds were added to the protein-peptide complex and the FP values were monitored over time at 37℃.

6. Isothermal Titration Calorimetry

LC3B was purified to the ITC buffer (50 mM HEPES pH 7.50) and degassed prior to measurement at 4°C. FITC-LBP2 were dissolved and diluted with the same buffer to the final concentrations of 200 μM. Titrations were performed using an auto-ITC200 titration calorimetric system (MicroCal) at 25°C. Data was analyzed with Origin 7.0 (Origin Lab) to determine the Kd value, △H, △S and stoichiometry.

7. Thermodynamic Stability Studies by Differential Scanning Fluorimetry (DSF)

Differential scanning fluorimetry assays were performed on a Quant Studio 6 Flex Real-Time PCR system (ABI). Final concentrations of 4 μM LC3B protein, 5× SYPRO orange (Invitrogen) and a series of diluted compounds were mixed in the thermal shift buffer (50 mM HEPES pH 7.5; 1 mM DTT) to the volume of 20 μL. Each reaction was heated from 25 to 95°C and all samples were tested in triplicate. Absolute qPCR Plate Seal (Thermo Scientific) were used to limit evaporation. Fluorescence signal of SYPRO orange dye was monitored and applied to determine the melting temperature (*Tm*) values of LC3B with Protein Thermal Shift Software Version 1.1 (ABI).

8. NMR Titration Assays

All NMR experiments were collected at 25°C on a four-channel Bruker Avance III 600 MHz spectrometer equipped with a TCI cryoprobe. Interactions between the protein LC3B and the compound DC-LC3in were monitored by 2D ^{1}H-^{15}N HSQC spectra using uniformly ^{15}N-labeled proteins at 0.1 mM and DC-LC3in with gradient concentrations. NMR data were processed by the program NMRPipe[7] and analyzed with the software Sparky (Goddard and Kneller, Sparky 3, University of California, San Francisco). Chemical shift perturbation values ($\triangle\delta_{avg}$) for ^{15}N and ^{1}H nuclei were derived from equation (1):

$$\Delta\delta_{avg} = \sqrt{((\Delta\delta_N/5)^2 + \Delta\delta_H^2)/2} \qquad (1)$$

where$\triangle\delta_N$ and$\triangle\delta_H$ represent the chemical shift perturbation value of the amide nitrogen and proton, respectively. The resonance assignments for LC3B were obtained from BMRB (accession number: 5958)[8].

9. Mass Spectrometry

Nano-HPLC-MS/MS analysis: The peptides were dissolved in solvent A (0.1% formic acid in 2% acetonitrile and 98% H_2O), then loaded onto a manually packed reverse phase C18 column (15 cm× 75 μm I.D., packed with C18 resin, 3 μm particle size, 90 Å, Dikma Technologies Inc., Lake Forest, CA) coupled to EASY-nLC 1000 system (Thermo Fisher Scientific, Waltham, MA). Peptides were eluted from 5% to 80% solvent B (0.1% formic acid in 90% acetonitrile and 10% H_2O) in solvent A at a flow rate of 300 nL/min. For 60 min gradient, the conditions were as follows: 8-32% B over 48 min, 32-48% B over 4 min, 48-80% over 4 min, and then held at 80% B for 4 min. The eluted peptides were analyzed by Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Parameters were as follows: For full MS spectra, the scan range was *m/z* 350 ~ 1,300 with a resolution of 120,000 at *m/z* 200. MS/MS acquisition was performed in top speed mode with 3 s cycle time. The resolution was 15,000 at m/z 200.Intensity threshold was 5,000, and maximum injection time was 35 ms. AGC target was set to 7,000, and the isolation window was 1 *m/z*. Ions with charge states 2+, 3+ and 4+ were sequentially fragmented by higher energy collisional dissociation (HCD) with normalized collision energy (NCE) of 32%. The dynamic exclusion duration was set as 30 s.

Analysis of MS data: Raw files were analysed by Mascot search engine (version 2.3.01) against UniProt Human database (88,817 sequences, updated on 07/12/2014). Methionine oxidation and three modifications of compounds were chosen as variable modifications, and cysteine alkylation by iodoacetamide was chosen as a fixed modification. Mass error for parent ion mass was ± 10 ppm with fragment ion as ± 0.5 Da. The protease was specified as trypsin with 2 maximum missing cleavages. Peptides assigned with a Mascot score lower than 20 were further discarded.

For the GSH reaction analysis

DC-LC3in-D5 (1mM in DMSO, 1mL) and GSH (5 mM in PBS buffer, 1mL) were mixed together and it was incubated for indicated time at 37℃. The mixture was analyzed by UPLC-MS (Waters Acquity UPLC H-class with Waters SQ Detector 2) after 2 h incubation. The analysis process lasted for 10 min with a gradient of 10% acetonitrile to 100% acetonitrile in 0.1% formic acid aqueous solution.

For the proteomic analysis

Sample preparation: the click reactions were conducted as Zheng B.H. described[9] and the pull down of probe-labelled proteins was performed as Chan P.Y. described[10].

Protein Digestion: 100 uL of 100 mM ammonium bicarbonate and trypsin (10ng/uL) was added to each sample which was then incubated at 37℃ for 16h. The tryptic peptide mixtures were collected by centrifugation at 14,000g for 10 min and desalted by C18 tip as previously described [11].

LC/MS Instruments and bioinformatics： Enzyme digests of peptide mixture were analyzed on the Easy nano-LC1000 system (Thermo Fisher Scientific) using a self-packed column (75μm × 150mm; 3 μm ReproSil-Pur C18 beads, 120 Å , Dr.Maisch GmbH, Ammerbuch, Germany) at a flow rate of 300 nL/min. The mobile phase A of RP-HPLC was 0.1% formic acid in water, and B was 0.1% formic acid in acetonitrile. The peptides were eluted using a gradient (2–90% mobile phase B) over a 60min period into a nano-ESI Q Exactive mass spectrometer (Thermo Fisher Scientific). The mass spectrometer was operated in data-dependent mode with each full MS scan (m/z 350 - 1500) followed by MS/MS for the 15 most intense ions with the parameters: ≥ +2 precursor ion charge, 2 Da precursor ion isolation window and 27 normalized collision energy of HCD. Dynamic Exclusion™ was set for 30 s. The full mass and the subsequent MS/MS analyses were scanned in the Orbitrap analyzer with R = 70,000 and R= 17,500, respectively.

Data Analysis: The MS data were analyzed using the software MaxQuant[12] (http://maxquant.org/, version 1.6.1.0). Oxidation (M, +15.99492 Da) was set as a variable modification. Proteins were identified by searching MS and MS/MS data of peptides against a decoy version of the Uniprot human database (2018/11/20 download, 73120 protein sequences). Trypsin/P was selected as the digestive enzyme with two potential missed cleavages. Data are available via ProteomeXchange with identifier PXD026874. After identifying proteins from MS results, we first filtered to assure that each protein in “Probe” group was derived from 2 or more unique peptide. Among remaining 1043 proteins, no zero intensity was observed in “Probe” ABPP group. The zero intensity in “Control” and “Probe+Competitor” groups was assigned as 100. After normal distribution and homogeneity of variance test, ANOVA or t-test was performed among groups. The adjusted p-values were adjusted by Benjamini and Hochberg method. Data cleaning and analysis was processed by R(3.6.1). Figure plotting and modification was processed by R(3.6.1) and Adobe Illustration. The original data file and R scripts are appended in supplementary materials.

10. Crystallization

The protein-compound complexes co-crystallization was set up for crystallization using the sitting vapour-diffusion method and a Gryphon LCP crystallization robot (National Center for Protein Science Shanghai, China) with SWISS 2-well plates. For coarse screens, the different combinations of LC3B (1-125, 2-119 and 1-120) and DC-LC3in or its derivatives were performed respectively.

The apo LC3B (2-119) crystals were grown by the sitting drop vapour-diffusion method as previously described [13].

11. Data Collection and Structure Determination

Crystals were cryo-protected with 18% additional glycerol and the well solution and then were rapidly frozen in liquid nitrogen. Data were collected at wavelength of 0.97853 Å at Shanghai Synchrotron Radiation Facility beamline BL19U1. Data were indexed and integrated with XDS and scaled with Aimless in CCP4 package. Initial phases were calculated by molecular replacement with PHASER using the LC3B (2-119) apo structure (PDB 3VTU) as the searching model. The initial models were built with COOT [14] manually and refined with PHENIX [15] package. The validation of final model was performed with MolProbity[16]. All structure figures were generated with PyMol[17].

12. In vitro lipidation assays

Protein purification and LC3B and GABARAP lipidation assays were performed as described[6b]. Lipids (DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; bIPI, bovine liver L-α-phosphatidylinositol from Avanti Polar Lipids, Inc) in chloroform were mixed with a composition of 60 mol% DOPC, 30 mol% DOPE, 10 mol% bIPI and dried under nitrogen gas stream. The resulting lipid film was further dried under vacuum for 2 h and hydrated in 50 mM HEPES pH7.5 and 100 mM NaCl for 1 h with vigorous mixing. Then, the lipid solution was bath-sonicated for 5 min, freeze-thawed 3 times and sonicated using a probe-sonicator, yielding small unilamellar vesicles (SUVs). D9 was mixed with 100 μM LC3B or GABARAP proteins at a ratio of 3:1, and D5 was mixed with LC3B or GABARAP at a ratio of 3:1, 1:1, or 1:0.33. The mixtures were incubated for 12 h at 37˚C and then diluted with an enzyme-containing solution to finally contain 1 μM ATG7, 1 μM ATG3, 1 μM ATG12–ATG5-ATG16N (11-43), 10 μM LC3B (or GABARAP), 30 μM D9 (or 30, 10, or 3.3 μM D5) and 1 mM SUVs in 50 mM HEPES pH7.5, 100 mM NaCl, 1 mM MgCl2, 1 mM TCEP. The reaction was initiated by adding 1 mM ATP and let proceed for 90 min at 37˚C. At each time point, an aliquot was removed from the reaction solution and mixed with an equal volume of 2 × SDS sample buffer. Samples were separated in 12.5% acrylamide Tris-

glycine SDS gels containing 6 M urea. Gels were stained and scanned as described above. Bands were normalized against the amount of unconjugated LC3B or GABARAP at t=0.

13. In vitro conjugation assays

The LC3B~ATG7 and GABARAP~ATG7 thioester conjugation assays were performed as follows: D5 and D9 were dissolved in DMSO at a concentration of 50 mM and mixed with 100 μM LC3B or GABARAP at a ratio of 3:1 (=D5 or D9:LC3B or GABARAP). The mixtures were incubated for 12 h at 37˚C and then diluted with ATG7-containing buffer such that the final solution contained 3 μM ATG7 and 7 μM LC3B or GABARAP, and 21 μM D5 or D9 in 50 mM HEPES pH7.5, 100 mM NaCl, 1 mM MgCl2, 1 mM TCEP buffer solution. The conjugation reaction was initiated by adding 1 mM ATP and let proceed for 120 seconds at room temperature. At each time point, an aliquot was removed from the reaction solution and mixed with an equal volume of 2 × LDS sample buffer with no reducing reagents (Thermo Fisher Scientific). Immediately after the final aliquot (for t=120 s) was removed, another aliquot was mixed with 2 × LDS sample buffer with β-mercaptoethanol. All samples were kept on ice until they were loaded onto 12% acrylamide bis-Tris pH6.5 gels. Electrophoresis was performed in MOPS pH 7.2 buffer at 4˚C. Gels were stained with Coomassie Brilliant Blue R-250 and scanned using Odyssey infrared scanner (Li-Cor). Gel bands were quantified using the Image Studio Lite software (Li-Cor) and normalized against the amount of ATG7 at t=0.

14. Cellular Thermal Shift Assay (CETSA)

For the cell lysate CETSA experiment, cultured HeLa cells were harvested and washed with PBS. After being diluted in PBS supplemented with protease inhibitor cocktail, the cell suspensions were freeze-thawed three times using liquid nitrogen. The soluble fraction was separated from the cell debris by centrifugation at 20,000 g for 15 min at 4°C. Cell lysates were collected, diluted and divided into two aliquots, treated with DC-LC3in or DMSO as control for 30 min at room temperature. Then each sample was divided into smaller aliquots and dispensed into 0.2 mL PCR tubes, heated for 3 min to 52.0-71.0°C followed by cooling for 3 min at 25°C. The lysates heated were centrifuged at 20,000 g for another 15 min at 4°C, and supernatants were analyzed by SDS-PAGE followed by western blot analysis.
In the intact cell assay, HeLa cells were seeded into 10 cm plates and treated with indicated compounds or DMSO for 6 hrs in the next day. Cells were harvested and washed with PBS containing protease inhibitor cocktail in order to remove excess compound. Equal amounts of cell suspensions were dispensed into 0.2 mL PCR tubes and heated as in lysate experiment. All heated cell samples were then freeze-thawed with liquid nitrogen three times. The soluble fractions were isolated and analyzed by western blot analysis as described above.

15. EGFP-LC3 Affinity Purification

HeLa cells overexpressing EGFP-LC3 were treated with DC-LC3in-D5 or DMSO for 12 hrs. Cells were harvested, washed in ice-cold PBS and lysed on ice in cell lysis buffer (CST, Cat# 9803) supplemented with protease inhibitor cocktail (Roche Diagnostics, Cat# 04693132001), 0.5% sodium deoxycholate (Sigma, Cat# D6750), 10 mM NAM (Sigma, Cat# 72340) and 5 μM TSA (J&K Scientific, 303595). The lysate was clarified by centrifugation at 20,000 g. The GFP-Trap_A beads (Chromotek, gta-20) were washed using the same lysis buffer and then added to the lysate to bind the EGFP-LC3 protein at 4°C for 2 hrs. The beads were washed extensively again and were subjected to SDS-PAGE followed by Coomassie staining to separate the target protein.

16. Immunoblotting

For immunoblotting analysis for LC3B, GABARAP and p62, HeLa cells were pre-treated with the indicated compounds for 16hrs before the stimulation of EBSS or CQ (chloroquine)for another 4hrs. Cells were then lysed by 2×SDS-PAGE sample loading buffer and boiled at 99°C for 10 min, separated by SDS-PAGE and subjected to western blot analysis. Antibodies used are rabbit anti-LC3B polyclonal antibody (Novus, NB100-2220), GABARAP antibody (Invitrogene, PA5-78366), p62/SQSTM1 antibody (Proteintech, 18420-1-AP) PON2 antibody (ABclonal, A14048), SCARB2 antibody (ABclonal, A9185), Flag antibody (CellSignalingTech, 14793S), EGFP antibody (Abmart, M20004s) and GAPDH Rabbit Polyclonal antibody (Proteintech, 10494-1-AP). HRP-conjugated Goat Anti mouse or rabbit IgG was used (BBI, D110087 or D11005).

16. Immunofluorescence

Coverslips (NEST) were placed in 24 well plates and then coated with 0.1% gelatin (Merck Millipore, ES-006-B) for 30 minutes. HeLa cells stably overexpressing TurboRFP-EGFP- GABARAP were seeded onto coverslips. After indicated treatment as described, cells

were immobilized by 4% paraformaldehyde (Servicebio, G1101) and washed with PBS buffer. DAPI (Invitrogene) was used in 0.2ug/ml to stain nucleus. The samples were protected by antifade mountant (Invitrogene, P36961) and analyzed by Leica TCS SPS CFSMP confocal laser scanning microscopy. The quantification is performed by ImageJ.

17. Transmission Electron Microscopy

HeLa Cells were pre-treated with DMSO, DC-LC3-D5(10 μM), DC-LC3in-D9(10 μM) for 16 hrs followed by the stimulation of EBSS for 4 hrs. Cells were harvested by scraper blade, washed and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h and then post-fixed with 1% osmium tetroxide in the same buffer for 2 h. Samples were dehydrated in a graded series of ethanol and embedded in Epon 812. Ultrathin sections were stained with 4% uranyl acetate and lead citrate and observed under an electron microscope Tecnai G2 Spirit at 120kV.

18. Pull-down Assays

EGFP pull-down assays were performed using HEK293T cells transiently expressing human EGFP-LC3 WT or EGFP-LC3 K49R protein and human FLAG-ATG7 or human ATG3-FLAG protein with different concentrations of DC-LC3in-D5 or DC-LC3in-D9. Whole cell lysates were collected in ice-cold lysis buffer (Cell Signaling Technology, 9803S) supplemented with complete protease inhibitor (Roche Diagnostics, Cat# 04693132001). Clarified cell lysates were obtained by centrifugation at 12,000 rpm for 15min followed by incubated with GFP-Trap A beads (Chromotek, gta-20) at 4°C for 2 hrs. The beads were washed extensively again and were subjected to SDS-PAGE to separate the target protein.

19. Statistical Analysis

For quantifications of immunoblotting, immunofluorescence and Transmission Electron Microscopy, student t tests were performed using GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA).

Supplemental Figures

Figure S1.

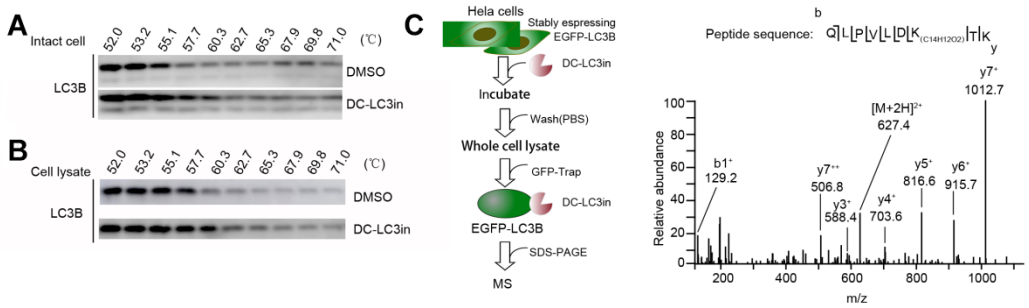


Figure S1. Binding of DC-LC3in to LC3 K49 inside cells. (A-B) Target engagements of DC-LC3in to LC3B inside Hela cells or in cell lysate using cellular thermal shift assays. Intact Hela cells (A) or cell lysate (B) were treated with DC-LC3in (200 μM) or DMSO as control. LC3B alone denatures around 55.1-57.7°C inside Hela cells and 57.7-60.3°C in the cell lysate. The additional of DC-LC3in stabilizes LC3B and brings to positive shift in the denature temperature by about 3-5°C, confirming the binding of DC-LC3in to LC3B inside Hela cells. (C) HPLC-nESI MS/MS mass spectrometry identified the modification of LC3B-Lys49 by DC-LC3in inside cells.

Figure S2.

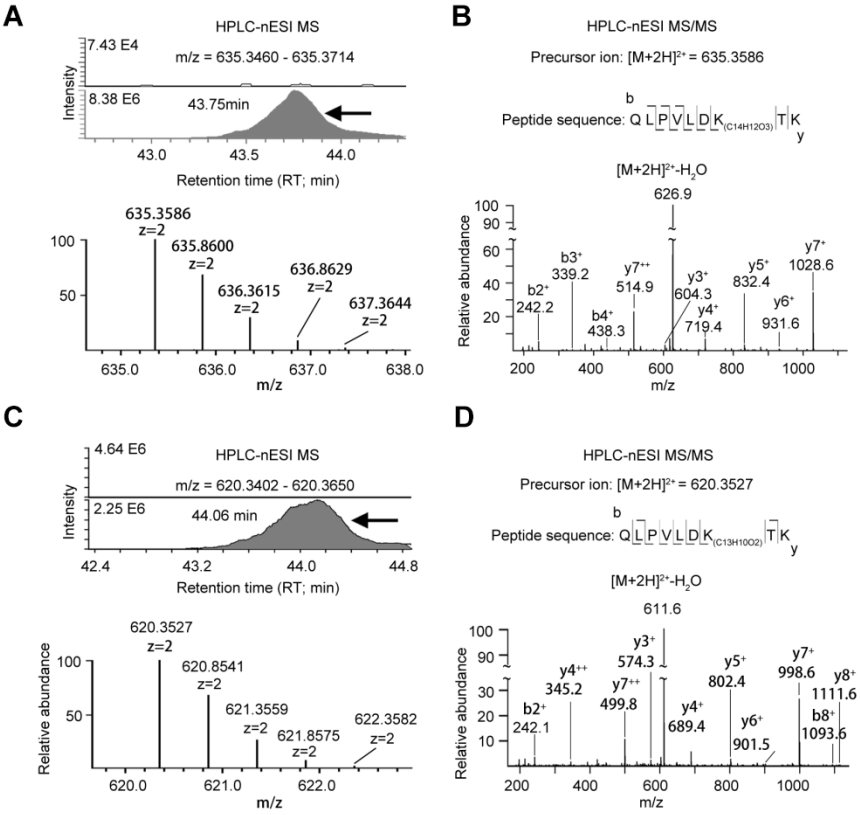


Figure S2. Active analogues of DC-LC3in also modified LC3B at Lys49. LC3B protein purified from E. coli was incubated with a1 (A-B), a2(C-D) or DMSO, subjected to SDS-PAGE and detected by HPLC-nESI MS/MS analysis. Both compounds modified the Lysine 49 of LC3B. The difference in peptide fragment mass weight between detected and theoretically predicted is corresponding to the chemical composition of C14H12O3 and C13H10O2 respectively, demonstrating the same way in which DC-LC3in and active derivatives reacting with LC3B.

Figure S3.

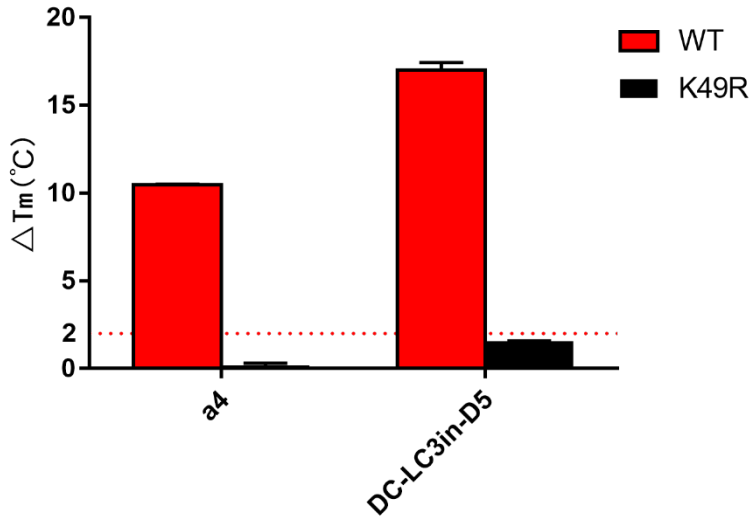


Figure S3. DC-LC3in derivative a4 and DC-LC3in-D5 showed no binding with LC3B K49R. The thermal shift values of WT or K49R LC3B incubated with a4 or DC-LC3in-D5 compared with DMSO control are shown in figure.

Figure S4.

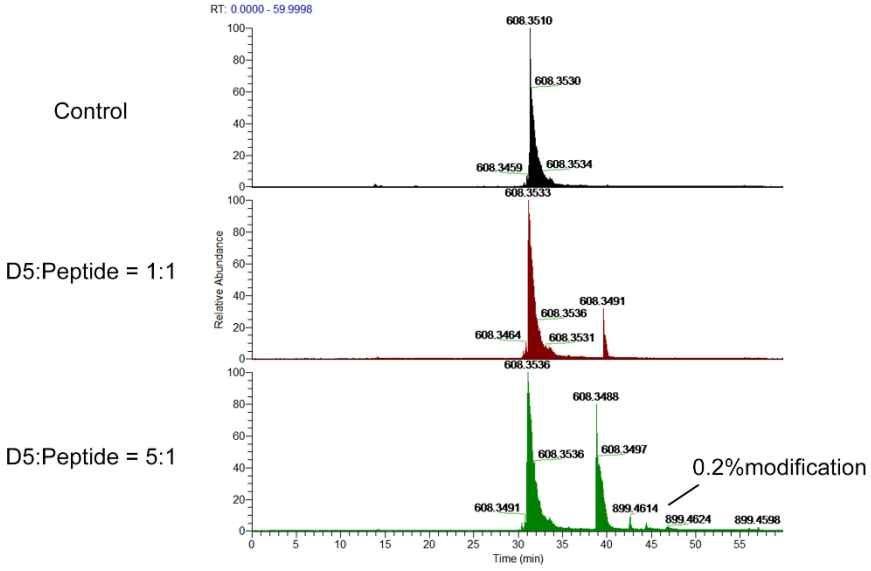


Figure S4. LC/MS result of peptide incubated with DC-LC3in-D5. Even in 5:1 ratio of DC-LC3in-D5:Peptide, only 0.2% peptide was modified by DC-LC3in-D5 (calculated by peak area).

Figure S5.

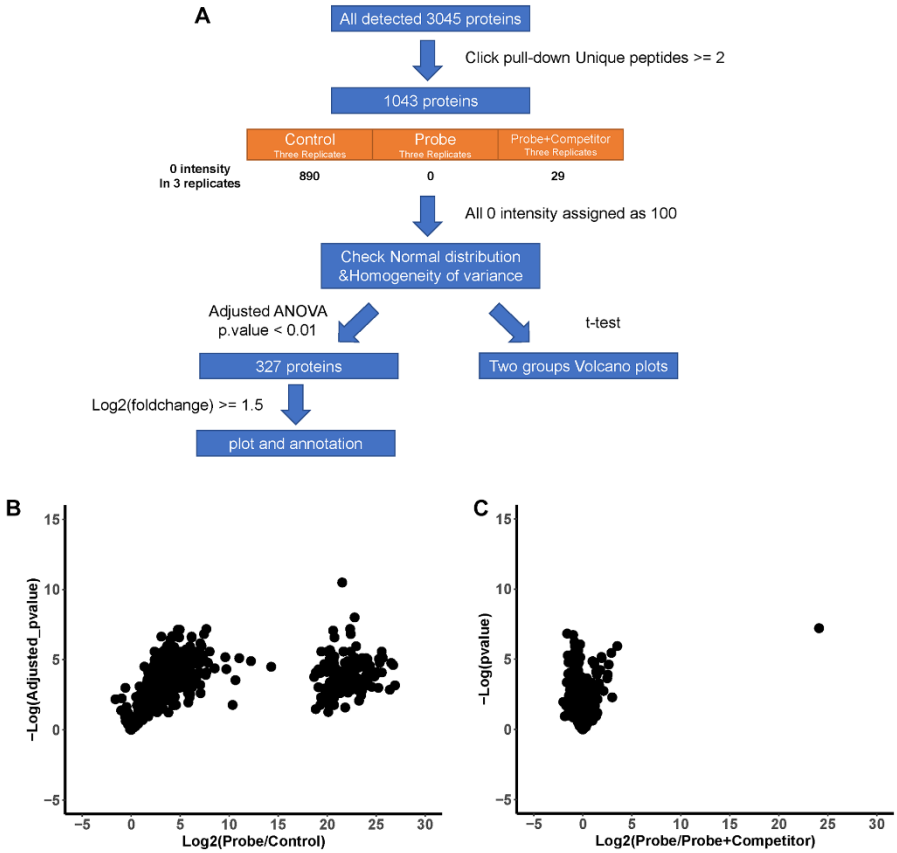


Figure S5. Downstream analysis method and volcano plots of ABPP MS data. (A) Downstream analysis method of ABPP MS data. (B) The volcano plots of Probe/Control groups. X-axis represented the Log2 intensity ratio. Y-axis represented ajusted p-values which were calculated by t-test and adjusted by Benjamini and Hochberg method. (C) The volcano plots of Probe/Competitor groups. X-axis represented the Log2 intensity ratio. Y-axis represented p-values which were calculated by t-test.

Figure S6.

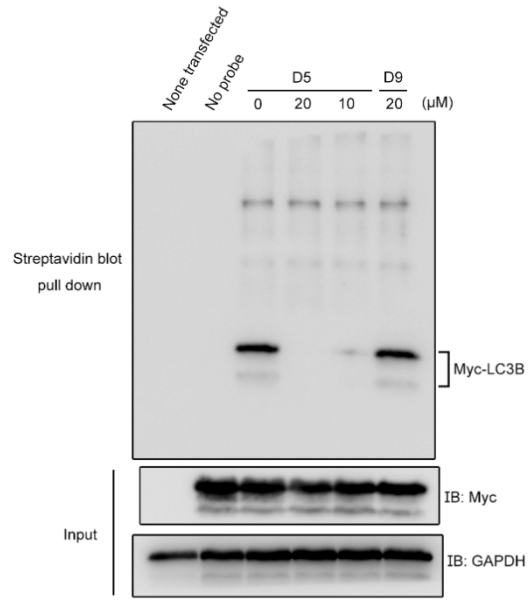


Figure S6. After overexpressing Myc-LC3B in HeLa cells, D5 or D9 was used to compete with ABPP probe to bind Myc-LC3B. Results showed that D9 failed to compete with ABPP probe.

Figure S7.

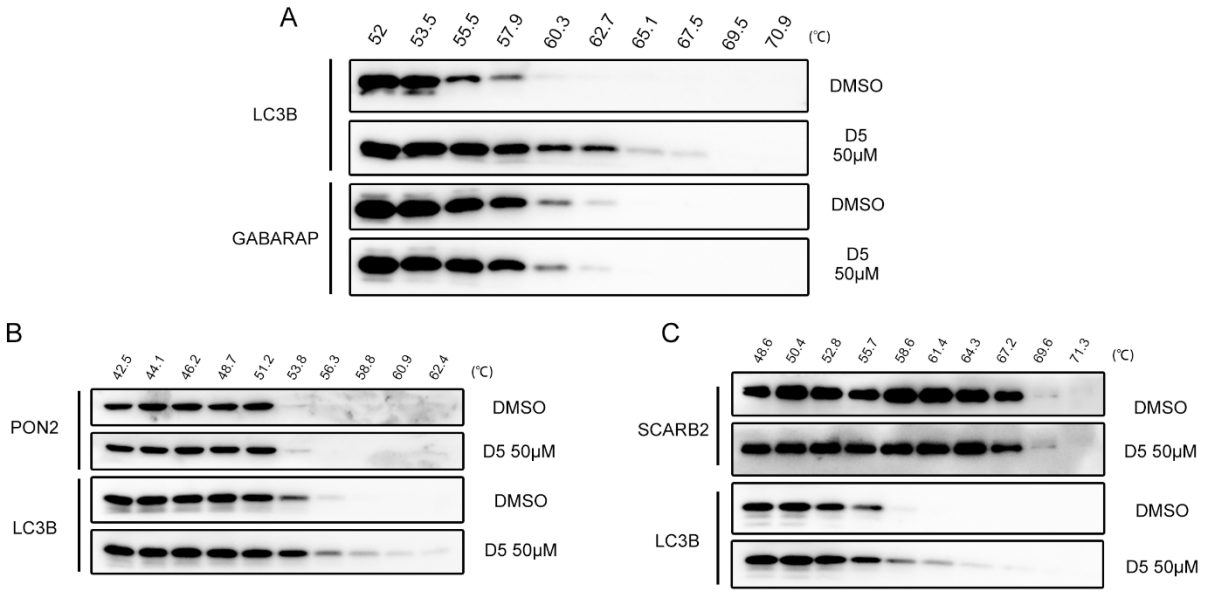


Figure S7. CETSA of DC-LC3in-D5. Intact Hela cells were treated with D5 (50 μM) or DMSO as control. (A) The additional of D5 stabilizes LC3B and brings to positive shift in the denature temperature by about 7°C. However, D5 did not stabilize GABARAP in same condition which indicates very weak binding between D5 and GABARAP in HeLa cells. (B-C) DC-LC3in-D5 did not bind with PON2 or SCARB2.

Figure S8.

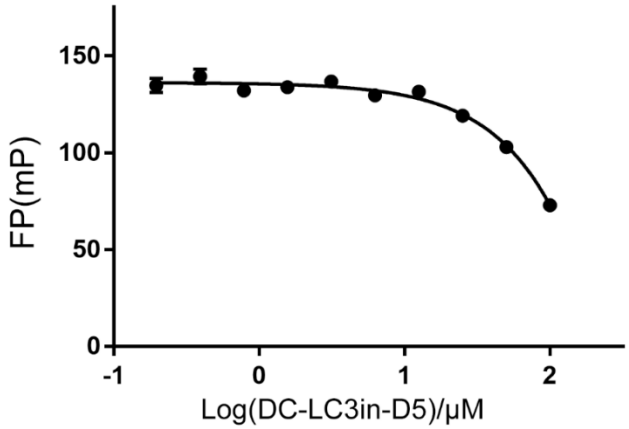


Figure S8. DC-LC3in-D5 shows weak inhibition on GABARAP in FP assay. In same condition as LC3B FP assay, DC-LC3in-D5 shows activity above 100μM.

Figure S9.

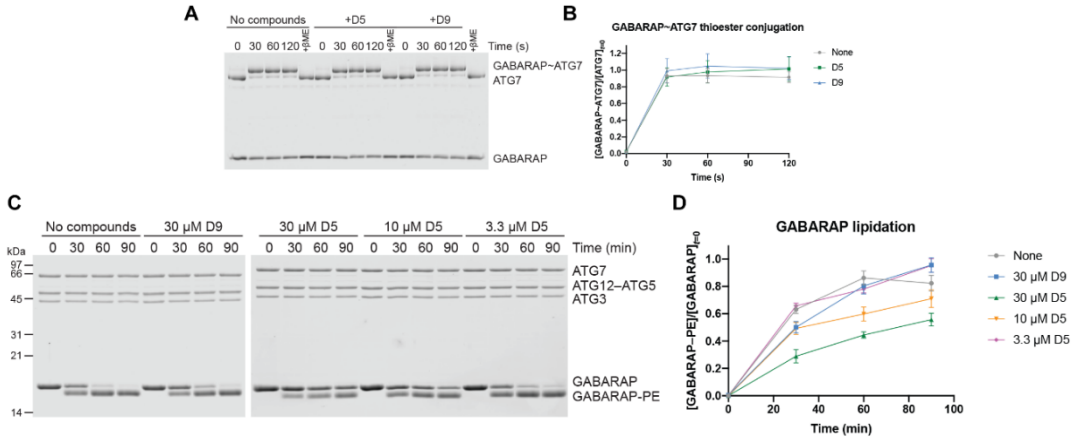


Figure S9. DC-LC3in-D5 showed no inhibition on ATG7-GABARAP conjugation and GABARAP lipidation. (A) DC-LC3in-D5 showed no inhibition on ATG7-GABARAP conjugation (D5 or D9:LC3B at a ratio of 3:1). (B) The ATG7-conjugation ratio of GABARAP is measured in independent experiments (right, ±s.d.; n=3). (C) DC-LC3in-D5 showed weak inhibition on GABARAP lipidation (GABARAP final concentration is 10μM). (D) The PE-modification ratio of GABARAP was measured in independent lipidation experiments (right, ±s.d.; n=6).

Supplemental Tables

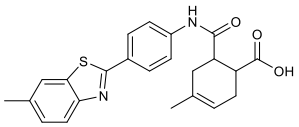
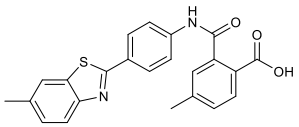
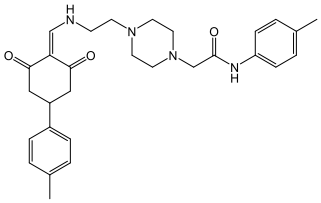
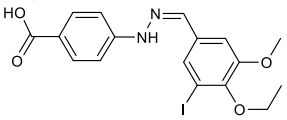
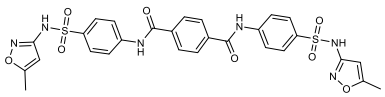
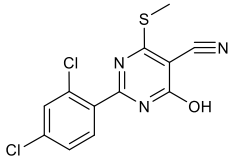
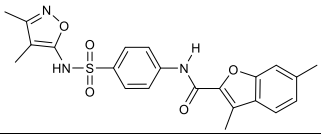
Table S1.

Table S1. Small molecules screening information of HTS performed to identify LC3B-LIR inhibitors.

Category	Parameter	Description
Assay	Type of assay	Fluorescence polarization (FP) competition assay
	Target	LC3B-LIR interaction
	Primary measurement	Detection of FP signal
	Key reagents	GST-LC3B, FITC-LBP2($p62_{333-347}$) peptide buffer: 50mM HEPES pH7.5, 1mM DTT, 0.1mg/mL BSA
	Assay protocol	Described in the Method Section
Library	Library size	1930
	Source	In house
Screen	Format	384-well, Corning 3575
	Concentration tested	250 μM, 2.5% DMSO
	Plate controls	NC: GST-LC3B, FITC-LBP2, PC: GST-LC3B, FITC-LBP2, LBP2
	Reagent/compound dispensing system	PerkinElmer, JANUS
	Detection instrument and software	PerkinElmer, EnVision
	Assay validation/QC	Z'=0.70; Mean FP for NC=197.6, SD =2.8 Mean FP for PC=30.95, SD =1.7
	Normalization	% inhibition = 100×(average of NC - sample)/(average of NC - average of PC)
Post-HTS analysis	Hit criteria	Inhibition rate >= 70%
	Hit rate	0.4%
	Confirmation of hit purity and structure	Compounds were repurchased from Specs and verified analytically

Table S2.

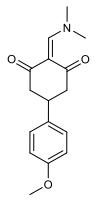
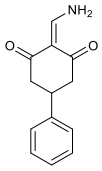
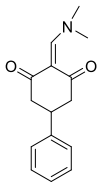
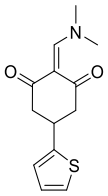
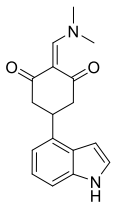
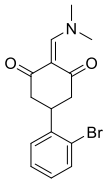
Table S2. LC3B modulators with IC_{50}<50μM from FP-based HTS.

SPECS ID	Structure	IC_{50} (μM)	△*T*m (°C)
AP-263/40778243		46	0
AP-263/40778244		50	0
AJ-292/14921009		3.06	8.52
AK-968/40641225		28	2.1
AN-329/40718893		28	1.8
AP-782/41885447		25	0
AR-318/42873262		47	0

For Differential Scanning Fluorimetry experiment validation, the molar ratio of protein LC3B and the compound was set as 1:10, with the concentration of 4 μM and 40 μM, respectively.

Table S3.

Table S3. DC-LC3in analogues.

Compound ID	Structure	IC_{50} (μM) Mean±SD （n=3）
a1		4.05±0.39
a2		25.7±1.74
a3		2.66±0.20
a4		5.35±1.10
a5		0.98±0.17
a6		0.56±0.17

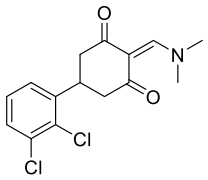
a7		0.27±0.10

Table S4.

Table S4. Diffraction and Refinement Statistics.

Wavelength	0.97853
Resolution range	30.4 - 1.599 (1.656 - 1.599)
Space group	P 21 21 21
Unit cell	35.108 54.766 60.792 90 90 90
Total reflections	204710 (20114)
Unique reflections	16025 (1562)
Multiplicity	12.8 (13.1)
Completeness (%)	99.69 (99.94)
Mean I/sigma(I)	36.72 (6.17)
Wilson B-factor	23.95
R-merge	0.03709 (0.469)
R-meas	0.0387 (0.4881)
CC1/2	1 (0.97)
CC*	1 (0.992)
Reflections used in refinement	16024 (1561)
Reflections used for R-free	770 (66)
R-work	0.1940 (0.2035)
R-free	0.2307 (0.2606)
CC(work)	0.962 (0.929)
CC(free)	0.908 (0.906)
Number of non-hydrogen atoms	1111
macromolecules	1015
ligands	19
Protein residues	122
RMS(bonds)	0.007
RMS(angles)	0.97
Ramachandran favored (%)	100
Ramachandran allowed (%)	0.00
Ramachandran outliers (%)	0.00
Rotamer outliers (%)	0.00
Clashscore	4.86
Average B-factor	33.50
macromolecules	32.75
ligands	43.79
solvent	40.93
Number of TLS groups	1

Table S5.

Table S5. Selectivity of DC-LC3in to the L-site of LC3B.

	Tm(℃)							
	WT	**F52W**	**L53W**	**V54W**	**V58W**	**E62W**	**I66W**	**I67W**
DMSO	59.82±0.02	61.20±0.04	52.91±0.02	46.60±0.05	64.07±0.01	58.64±0.03	57.46±0.22	49.16±0.10
DC-LC3in	70.72±0.07	70.90±0.13	53.40±0.07	45.41±0.03	65.12±0.07	59.66±0.03	68.27±0.04	49.93±0.11
LBP2	68.02±0.04	65.70±0.25	54.97±0.08	50.72±0.02	71.90±0.18	69.34±0.07	66.42±0.07	60.37±0.12
	ΔTm(℃)							
	WT	**F52W**	**L53W**	**V54W**	**V58W**	**E62W**	**I66W**	**I67W**
DMSO	—	—	—	—	—	—	—	—
DC-LC3in	10.21	9.70	—	—	—	—	10.81	—
LBP2	8.20	4.50	2.06	4.12	7.83	10.70	8.96	11.21

The molar ratio of protein LC3B and the compound was set as 1:12.5, with the concentration of 4 μM and 50 μM respectively.

Table S6.

Table S6. GI_{50} value of DC-LC3in-D5 in various cancer cell lines.

Cell lines	Tumor types	Detection Assay	GI_{50} (μM)
Hela	Cervical cancer	Alamar Blue	>100
Capan-1	Pancreatic cancer	Alamar Blue	>100
H1299	Non-small-cell lung cancer	Alamar Blue	>100
HCT116	Colon cancer	Alamar Blue	>100
U266B1	Multiple myeloma	CellTiter-Glo	>100

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Chemical Synthesis and Optimization: BZ, TL, YX, BingZ
Protein Mass Experiments: MZ, MT
NMR Experiments: NZ, YW
Visualization: SF, LY, WW, CO
Supervision: KC, HJ, HD, LL, JL, ZC, HT, PX, JH, ZY, YX, YD, TO, CL
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Writing—review & editing: SF, YZ, TO, CL