Electronic Supplementary Material (ESI) for

Chemoproteomic Profiling Reveals Celastrol as a Potential Modulator of Cholesterol Signaling

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Table S1 Full list of celastrol targets in MCF-7 cells. (Please see accompanying Excel file)

Table S2 Previously reported celastrol targets and their enrichment and competition ratios in this study. *The data in the last column (AVE-SC) represent the propensity of a protein to be 'nonspecific' in streptavidin pull-down experiments, which were obtained online from CRAPome 2.0 (https://reprint-apms.org).

ID	Target [Ref]	Log_2-$R_{Enrichment}$	Log_2-$R_{Competition}$	AVE-SC*
P07900	**HSP90AA1** [1]	3.20	3.01	20.53
P08238	**HSP90AB1** [1]	3.24	2.95	33.27
Q16543	**CDC37** [2]	3.18	2.86	1.50
Q06830	**PRDX1** [3]	2.65	2.09	40.20
P32119	**PRDX2** [4]	2.18	1.70	10.13
Q01518	**CAP1** [5]	2.17	1.84	3.20
P49327	**FASN** [6]	2.60	2.13	180.43
P18031	**PTPN1** [7]	2.88	2.63	1.50
P07355	**ANXA2** [8]	2.38	2.12	41.79
P07437	**TUBB** [8]	2.46	2.01	58.00
P68104	**EEF1A1** [8]	2.36	2.27	39.20
P09429	**HMGB1** [9]	2.81	2.66	2.00
P00390	**GSR** [10]	2.45	2.12	12.11
P78417	**GSTO1** [10]	3.20	2.83	6.50
P07237	**P4HB** [10]	3.48	3.13	10.86

References for Table S2:

[1] Sekiya et al. Characterization of celastrol to inhibit hsp90 and cdc37 interaction. *J. Biol. Chem.* **2009**, *284*, 35381–35389.

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syndrome in mice. *Acta Pharm. Sin. B* **2021**, *11*, 1200–1212.
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[10] Zhou et al. Competitive profiling of celastrol targets in human cervical cancer HeLa cells via quantitative chemical proteomics. *Mol. BioSyst.* **2016**, *13*, 83–91.

Table S3 Celastrol targets included in the term “Proteasome” in KEGG functional pathway analysis. These proteins were highlighted as orange dot within the scattered plot of Figure 1D.

ID	Target	Log_2-$R_{Enrichment}$	Log_2-$R_{Competition}$
P62191	**PSMC1**	2.67	2.21
P35998	**PSMC2**	2.92	2.41
P17980	**PSMC3**	2.76	2.19
P43686	**PSMC4**	2.63	2.08
P62195	**PSMC5**	2.24	1.65
P62333	**PSMC6**	2.17	1.79
Q99460	**PSMD1**	2.76	2.37
O00232	**PSMD12**	2.57	2.26
Q9UNM6	**PSMD13**	2.86	2.28
O00487	**PSMD14**	2.36	2.19
Q13200	**PSMD2**	2.90	2.48
O43242	**PSMD3**	2.36	1.93
P55036	**PSMD4**	2.47	1.92
Q15008	**PSMD6**	2.72	2.50
Q06323	**PSME1**	2.53	1.97
Q9UL46	**PSME2**	3.12	2.71
P61289	**PSME3**	2.36	2.12
P25786	**PSMA1**	2.49	2.15
P25787	**PSMA2**	2.00	1.84
P25788	**PSMA3**	2.53	2.38
P28066	**PSMA5**	2.66	2.52
P60900	**PSMA6**	2.27	2.18
O14818	**PSMA7**	2.49	2.13
P49720	**PSMB3**	2.83	2.44
P28072	**PSMB6**	2.40	1.74

Table S4 Celastrol targets included in the term “Chaperone” in KEGG functional pathway analysis.

ID	Target	Log_2-$R_{Enrichment}$	Log_2-$R_{Competition}$
P61221	**ABCE1**	2.90	2.28
O95817	**BAG3**	2.06	1.80
P46379	**BAG6**	2.49	2.11
P31689	**DNAJA1**	2.95	2.61
P25685	**DNAJB1**	2.80	2.57
Q99615	**DNAJC7**	2.89	2.37
Q02790	**FKBP4**	2.81	2.32
Q99497	**PARK7**	2.23	2.06
Q12931	**TRAP1**	2.55	1.92
P27797	**CALR**	2.13	1.96
Q16543	**CDC37**	3.18	2.86
P78371	**CCT2**	2.21	1.94
P49368	**CCT3**	2.88	2.52
P50991	**CCT4**	2.43	2.03
P48643	**CCT5**	2.69	2.34
P40227	**CCT6A**	2.56	2.13
Q99832	**CCT7**	2.68	2.30
P50990	**CCT8**	2.48	2.05
Q14019	**COTL1**	2.83	2.58
Q9BS26	**ERP44**	2.29	1.59
P07900	**HSP90AA1**	3.20	3.01
P08238	**HSP90AB1**	3.24	2.95
P14625	**HSP90B1**	2.78	2.45
P0DMV9	**HSP90A1A**	2.82	2.44
O95757	**HSPA4L**	2.20	2.03
P11142	**HSPA8**	3.05	2.69
P38646	**HSPA9**	2.60	2.28
P04792	**HSPB1**	2.68	2.53
P10809	**HSPD1**	2.75	2.46
Q9Y4L1	**HYOU1**	2.61	2.26
P07237	**P4HB**	3.48	3.13
Q15084	**PDIA6**	2.63	2.24
O43765	**SGTA**	2.63	1.99
P50502	**ST13**	2.43	2.14
P17987	**TCP1**	2.49	2.14
Q9BTW9	**TBCD**	2.38	1.71
Q9H3U1	**UNC45A**	3.01	2.48

Table S5 Celastrol targets included in the term “Glycolysis” in KEGG functional pathway analysis.

ID	Target	Log_2-$R_{Enrichment}$	Log_2-$R_{Competition}$
P11766	**ADH5**	2.25	1.77
P30837	**ALDH1B1**	2.77	1.66
P49419	**ALDH7A1**	2.12	1.91
P49189	**ALDH9A1**	2.62	2.10
P04075	**ALDOA**	2.62	2.45
P09972	**ALDOC**	2.81	2.29
P09622	**DLD**	2.32	2.04
P06733	**ENO1**	2.48	2.21
P09467	**FBP1**	2.74	1.99
O00757	**FBP2**	2.11	1.84
P06744	**GPI**	3.13	2.79
P19367	**HK1**	2.59	2.05
P00338	**LDHA**	2.56	2.16
Q16822	**PCK2**	2.72	2.31
P17858	**PFKL**	2.52	1.93
P08237	**PFKM**	2.32	1.79
Q01813	**PFKP**	2.81	2.30
P00558	**PGK1**	2.63	2.24
P18669	**PGAM1**	2.12	1.90
P11177	**PDHB**	2.37	2.06
P14618	**PKM**	2.76	2.26
P60174	**TPI1**	2.52	2.36

Table S6 Celastrol targets included in the term “Lipid Metabolism” in KEGG functional pathway analysis. These proteins were highlighted as blue dot within the scattered plot of Figure 1D.

ID	Target	Log_2-$R_{Enrichment}$	Log_2-$R_{Competition}$
P80303	**NUCB2**	3.45	2.51
P05141	**SLC25A5**	3.29	2.03
Q5K4L6	**SLC27A3**	3.14	2.31
Q8NF37	**LPCAT1**	3.06	2.39
P50851	**LRBA**	2.94	2.26
P46977	**STT3A**	2.86	1.53
O15269	**SPTLC1**	2.86	2.04
P30084	**ECHS1**	2.80	2.39
P49748	**ACADVL**	2.79	2.26
Q6P1A2	**LPCAT3**	2.73	1.65
P07602	**PSAP**	2.70	1.80
P05023	**ATP1A1**	2.70	2.27
Q9HDC9	**APMAP**	2.68	1.62
Q9Y679	**AUP1**	2.68	2.10
O95470	**SGPL1**	2.68	2.03
P09110	**ACAA1**	2.65	2.53
P53396	**ACLY**	2.63	1.94
P40939	**HADHA**	2.62	1.65
P13798	**APEH**	2.61	2.08
P51659	**HSD17B4**	2.58	2.12
Q8TC12	**RDH11**	2.56	2.07
Q16698	**DECR1**	2.53	2.08
P11413	**G6PD**	2.51	2.20
Q96QD8	**SLC38A2**	2.49	1.84
Q8TCJ2	**STT3B**	2.48	1.71
Q9HD45	**TM9SF3**	2.47	1.91
O95573	**ACSL3**	2.00	1.66
Q99714	**HSD17B10**	2.45	1.79
P53007	**SLC25A1**	2.45	2.07
P19174	**PLCG1**	2.44	2.11
Q9NZ01	**TECR**	2.40	1.59
Q96G23	**CERS2**	2.38	1.66
P30041	**PRCX6**	2.38	1.99
Q92544	**TM9SF4**	2.33	2.01
P09622	**DLD**	2.31	2.04
Q96N66	**MBOAT7**	2.28	2.28
Q8N2K0	**ABHD12**	2.23	2.27
P55084	**HADHB**	2.22	1.91
P42765	**ACAA2**	2.21	1.84
Q6P1M0	**SLC27A4**	2.02	2.01
P36957	**DLST**	2.15	1.88
P29373	**CRABP2**	2.13	2.49
Q9P035	**HACD3**	2.11	1.56
P51149	**RAB7A**	2.08	1.59

Table S7 Celastrol targets included in the term “Cholesterol Biosynthesis” in KEGG functional pathway analysis. These proteins were highlighted as red diamond within the scattered plot of Figure 1D.

ID	Target	Log_2-$R_{Enrichment}$	Log_2-$R_{Competition}$
P07900	**DHCR24**	2.89	2.39
P08238	**DHCR7**	2.92	2.45
P78417	**LSS**	2.53	2.09
P07237	**CYP51A1**	2.97	1.91
Q16543	**TM7SF2**	2.70	2.38
P49327	**LBR**	2.50	1.80
Q06830	**MVD**	2.30	1.90
P14324	**FDPS**	2.71	2.33
P00390	**FDFT1**	2.61	1.89
P68104	**SQLE**	2.63	1.84

Table S8 Celastrol targets related to (A) metabolism, (B) transportation and (C) receptor of steroids. These proteins were highlighted as red dot in Figure 1D.

	ID	Target	Log_2-$R_{Enrichment}$	Log_2-$R_{Competition}$	Function / Interaction	References
Enzymes	Q6PIU2	**NCEH1**	2.50	2.43	Hydrolysis of cholesterol ester in macrophages	[1]
	Q9BWD1	**ACAT2**	2.50	1.66	Involved in the biosynthetic pathway of cholesterol	[2]
Transporters	O15118	**NPC1**	2.56	2.19	Intracellular cholesterol transporter	[3][17]
	P07099	**EPHX1**	2.97	2.32	Cellular uptake of bile acid	[4][18]
	P04114	**APOB**	2.67	1.95	Serum bile acid transporter	[5][18]
	P22059	**OSBP1**	3.15	2.01	Sterol transporter activity and oxysterol binding	[6]
	Q9BZF1	**OSBPL8**	2.77	2.30	Sterol transporter activity and oxysterol binding	[7]
	Q9BQE5	**APOL2**	3.02	1.97	Cholesterol transporter activity	[8]
	P08133	**ANAX6**	2.62	2.20	Cholesterol transporter activity	[9]
Binding/ regulating proteins	P05556	**ITGB1**	2.46	2.20	One of sensor proteins for tauroursodeoxycholic acid	[10][18]
	O00264	**PGRMC1**	2.94	2.16	membrane components of progesterone receptors	[11][17]
	O15173	**PGRMC2**	3.18	1.82	membrane components of progesterone receptors	[11][17]
	P12236	**SLC25A6**	2.94	2.24	Cholesterol-regulated ADT/ATP carrier	[12][17][18]
	Q3SXM5	**HSDL1**	3.21	2.38	Inactive form of metabolic enzyme of cholesterol	[13][17]
	P02768	**ALB**	2.01	1.61	Bile acid binding protein	[14][18]
	Q00341	**HDLBP**	2.46	2.15	Protecting cells from over-accumulation of cholesterol	[15]
	P19367	**HK1**	2.59	2.05	Cholesterol binding protein in mitochondria	[16][17]

References for Table S7:

[11] Sekiya et al. The role of neutral cholesterol ester hydrolysis in macrophage foam cells. *J. Atheroscler Thromb.* **2011**, *18*, 359–364.

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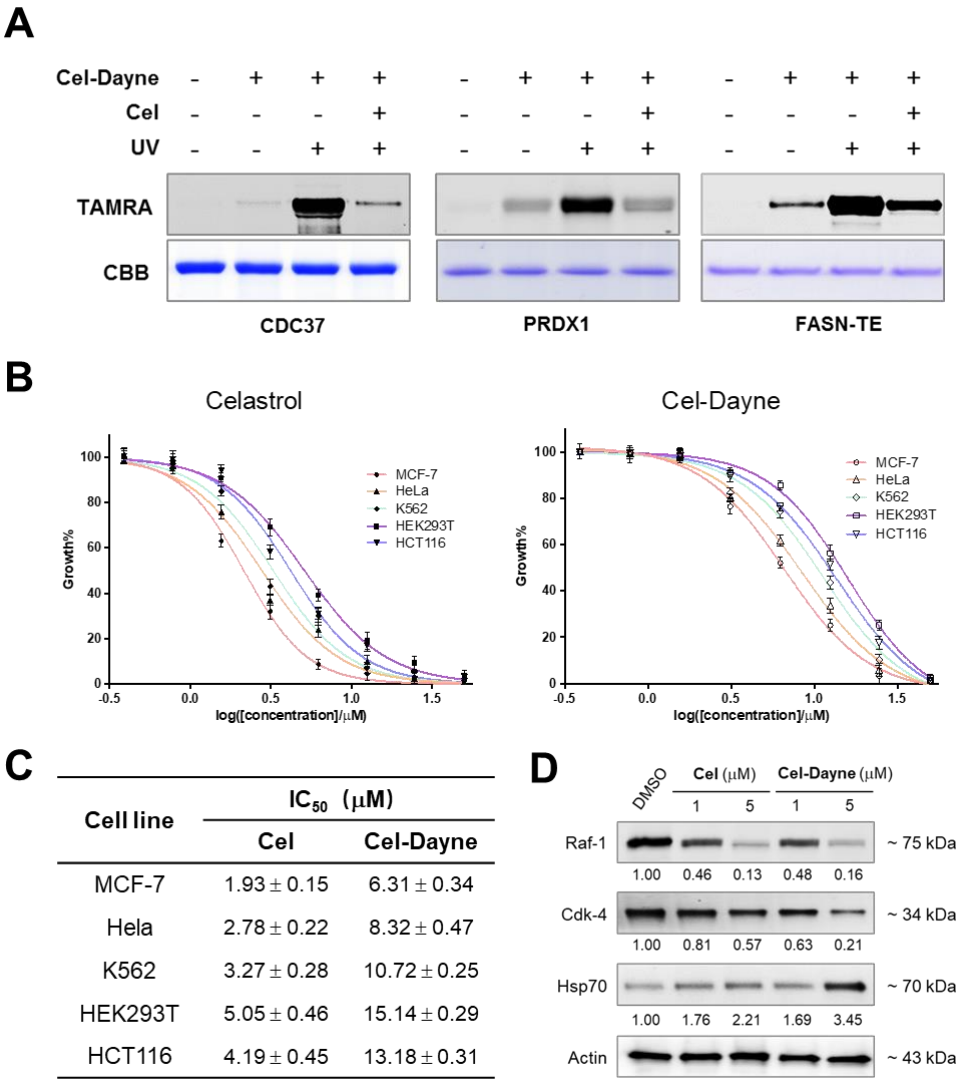


Cell line	IC_{50} (μM)	
	Cel	Cel-Dayne
MCF-7	1.93 ± 0.15	6.31 ± 0.34
Hela	2.78 ± 0.22	8.32 ± 0.47
K562	3.27 ± 0.28	10.72 ± 0.25
HEK293T	5.05 ± 0.46	15.14 ± 0.29
HCT116	4.19 ± 0.45	13.18 ± 0.31

Figure S1 (A) *In vitro* labeling of recombinant proteins of previously reported celastrol targets (CDC37, PRDX1, and FASN-TE) with Cel-Dayne; (B) Cell proliferation inhibitory effect of celastrol (Cel) and Cel-Dayne towards various cancer cell lines upon 48 h treatment; (C) IC_{50} values of celastrol (Cel) and Cel-Dayne towards various cell lines; (D) Effect of Cel and Cel-Dayne on the expression of Hsp90 client proteins (Raf-1 and Cdk-4) and heat shock marker Hsp70. MCF-7 cells were treated with Cel or Cel-Dayne as indicated for 18 h. The results are a representative of three biologically independent experiments. The densitometry analysis of the proteins was normalized to ACTIN.

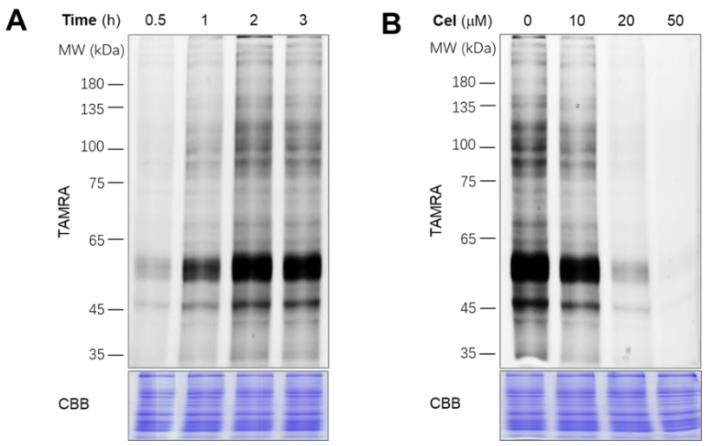


Figure S2 Cel-Dayne labeling of living MCF-7 cells. (A) Time-course labeling of MCF-7 cells with 10 μM Cel-Dayne; (B) Competition of Cel-Dayne labeling of MCF-7 cells with various concentrations of celastrol (Cel). Cells were pretreated with celastrol for 1 h, followed by Cel-Dayne labeling (10 μM, 2 h).

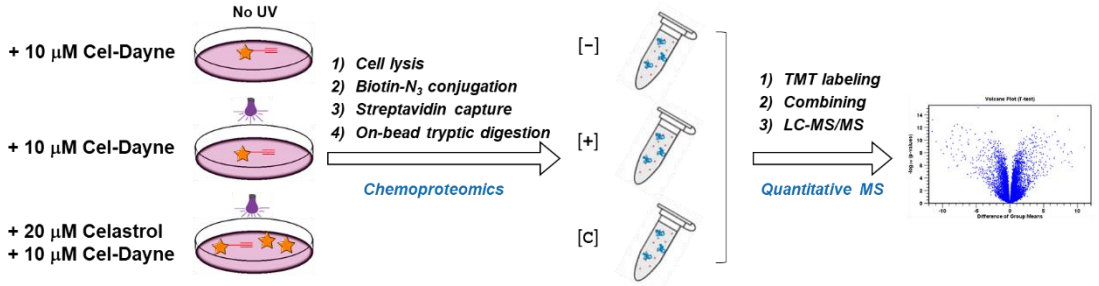


Figure S3 Workflow of quantitative mass spectrometry-based target profiling of celastrol.

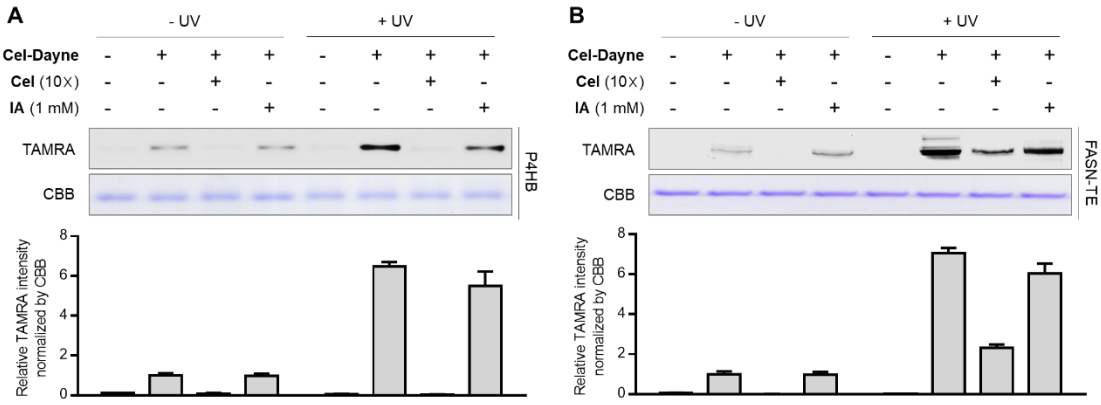


Figure S4 *In vitro* Cel-Dayne (10 μM) labeling of (A) recombinant protein disulfide isomerase (P4HB); and (B) Fatty acid synthase thioesterase domain (FASN-TE) proteins with and without UV irradiation. Free celastrol (100 μM) or iodoacetamide (1 mM) were used as competitors. Densitometry results for labeling was normalized by coomassie blue staining. Data represent mean values of three random points within each band.

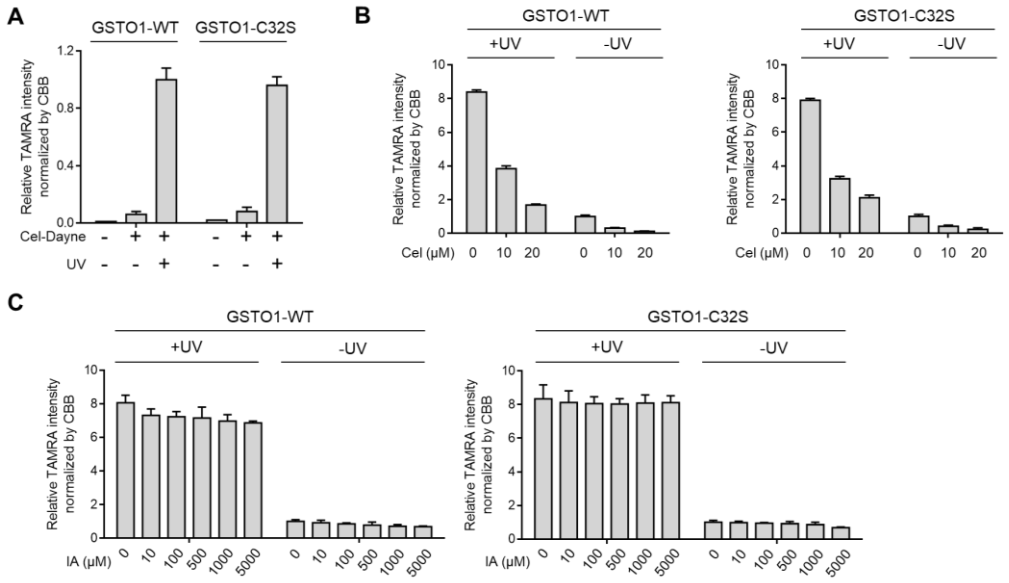


Figure S5 Densitometry results for GSTO1 labeling normalized by coomassie blue staining. (A) Densitometry for Fig. 2C; (B) Densitometry for Fig. 2D; (C) Densitometry analysis for Fig. 2C. Data represent mean values of three random points within each band.

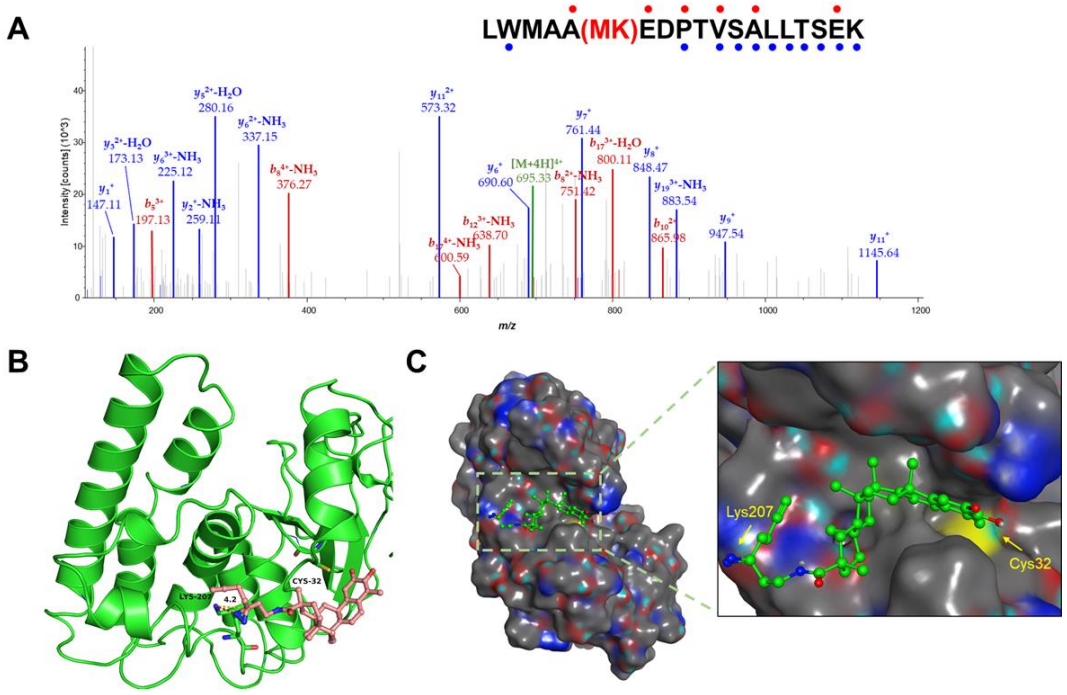


Figure S6 (A) Secondary mass spectrometry revealed Met206 or Lys207 as potential diazirine-labeled sites of GSTO1; (B) Docking of celastrol into the GSH binding site of GSTO1 (PDB ID code: 1EEM); (C) Surface view of celastrol-GSTO1 docking model.

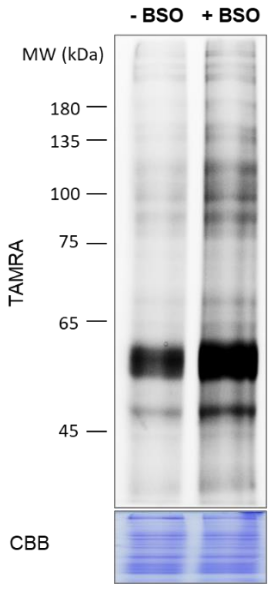


Figure S7 The effect of BSO on Cel-Dayne labeling of MCF-7 cells. Cells were pretreated with 100 μM BSO for 24 h prior to Cel-Dayne labeling (10 μM, 2 h).

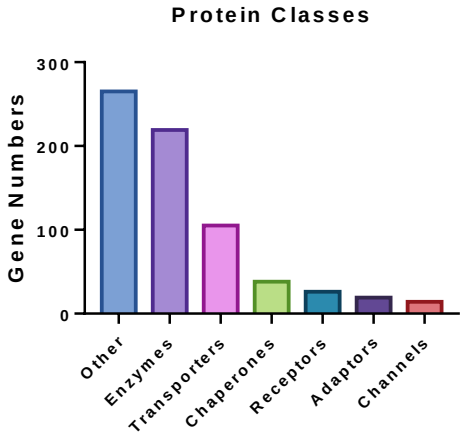


Figure S8 Geno Ontology (GO) molecular function analysis suggested celastrol binding proteins mainly belong to the terms of enzymes and transporters.

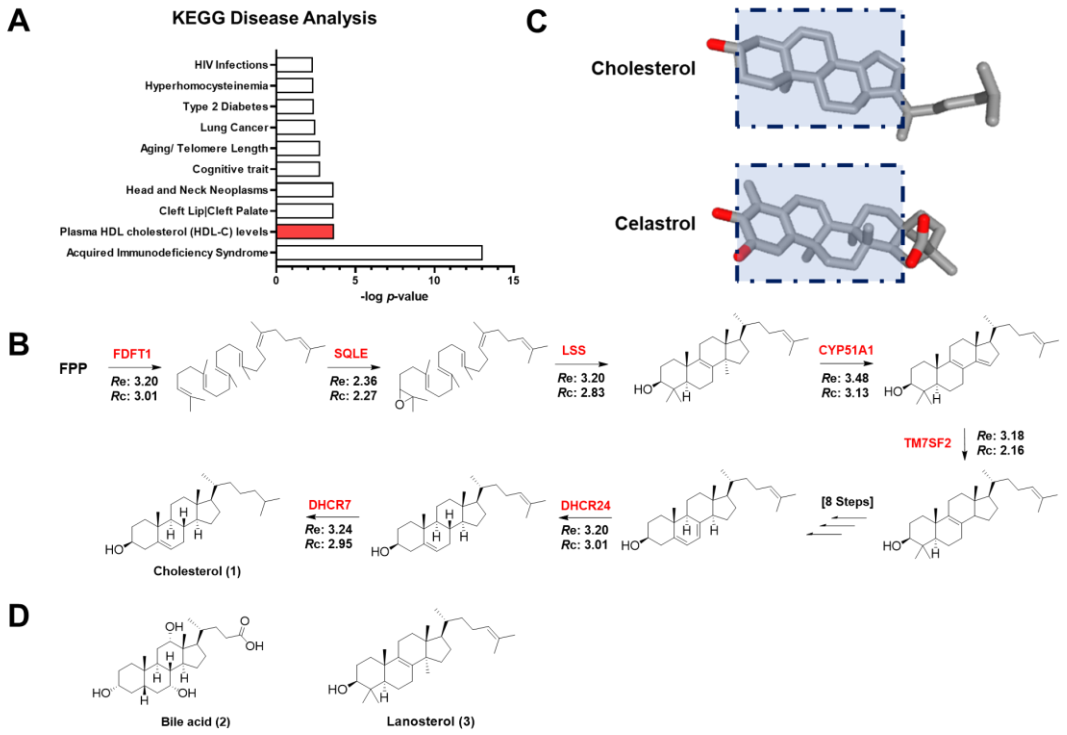


Figure S9 (A) Top 10 categories of KEGG diseases analysis; (B) Celastrol specifically targets multiple enzymes involved in cholesterol biosynthetic pathway; (C) The structure similarity between celastrol and cholesterol; (D) Structures of bile acid (**2**) and lanosterol (**3**) used in competitive labeling experiment in Fig. 3D.

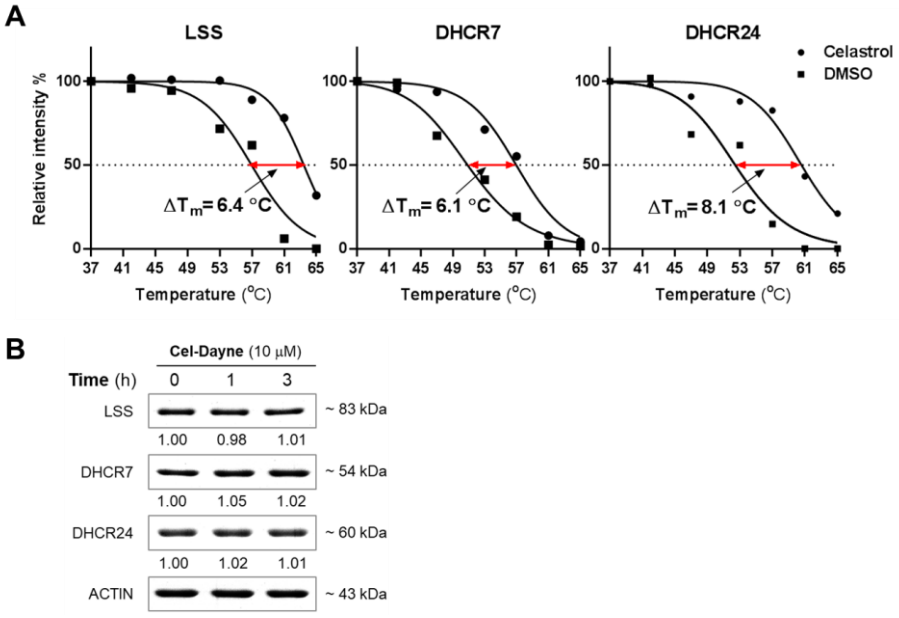


Figure S10 (A) The band intensities in Fig. 4B were measured and fitted to obtain apparent ΔTm values; (B) Cel-Dayne (10 μM, up to 3 h) exhibited no effect on the protein levels of LSS, DHCR7, and DHCR24 in MCF-7 cells. The results are a representative of three biologically independent experiments. The densitometry analysis of the proteins was normalized to ACTIN.

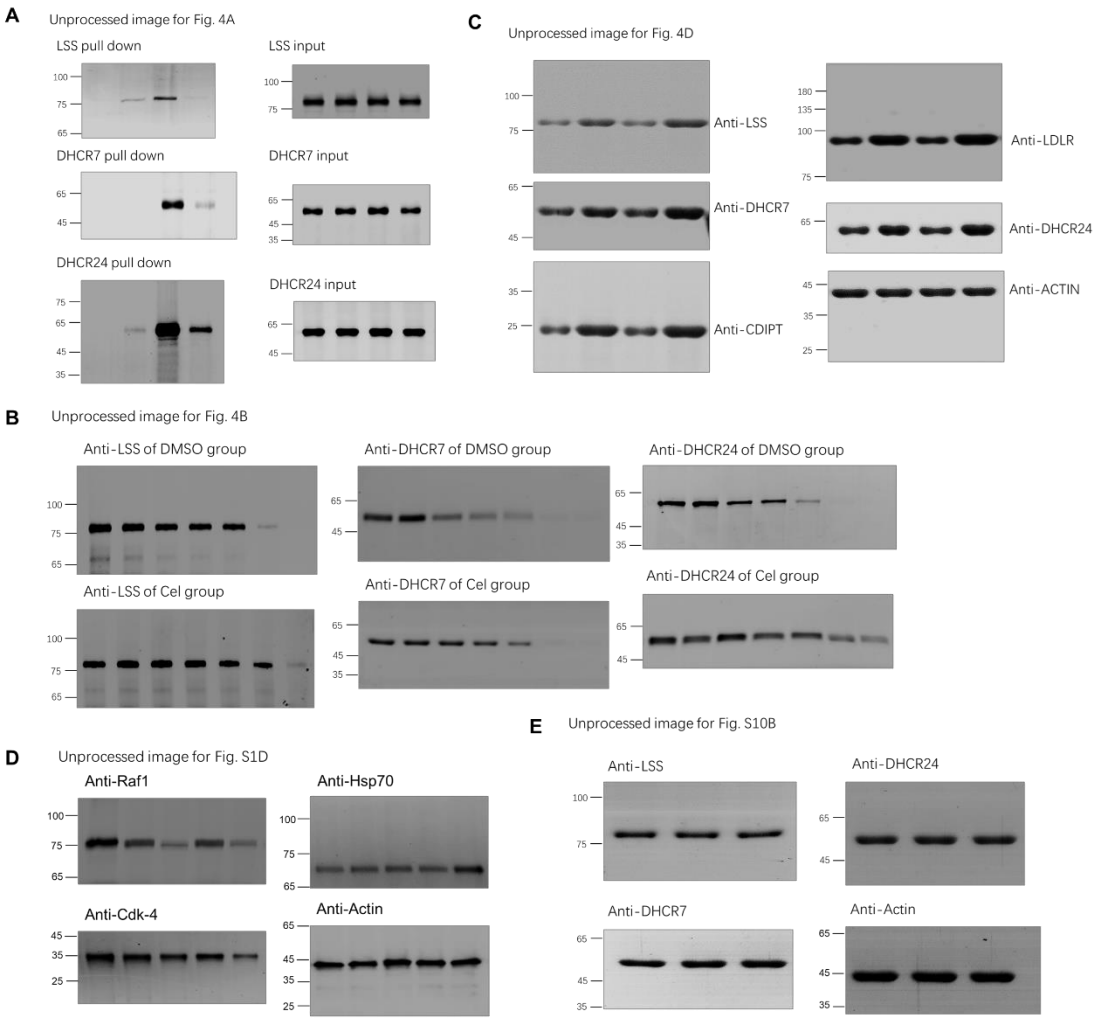


Figure S11 Unprocessed images of immunoblots for (A) Fig. 4A; (B) Fig. 4B; (C) Fig. 4D; (D) Fig. S1D; and (E) Fig. S10B.

2. Procedures for Biological Experiments

2.1 Cell proliferation assay

All cell lines (MCF-7, HCT116, HeLa, HEK293T, and K562) were ordered from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). MCF-7, HCT116, HEK293T, and HeLa cells were grown in DMEM (Gibco) supplemented with 1% L-Glutamine, 1% Penicillin-Streptomycin and 10% Fetal Bovine Serum (FBS, Gibco). K562 cells were grown in RPMI medium (Gibco) supplemented with 1% L-Glutamine, 1% Penicillin-Streptomycin and 10% Fetal Bovine Serum (FBS, Gibco). The culture incubator set is 37 °C with 5% CO_2. Cells cultured for three passages were diluted in culture medium to 1×10^6 cells/mL. 100 μL of cell suspension were seeded to each well (10^5 cells/well) of 96-well plate and incubated at 37 °C overnight. Cells in 96-well plate were treated with various concentrations of compounds or DMSO for 48 h in a 37 °C incubator. Cell viability was assessed by CellTiter-Glo® Luminescent Kit (Promega). Three biologically independent experiments were carried out and data analysis was performed with GraphPad Prism 7.0.

2.2 Labeling of recombinant proteins with Cel-Dayne

Recombinant proteins were diluted to a final concentration of 0.1 mg/mL in PBS and incubated with Cel-Dayne either with or without competitors as indicated at room temperature. Samples were irradiated with a Spectroline EA-180 UV light (365 nm, 8 Watt) on ice for 10 minutes. Each of 20 μL protein samples were added with 1% SDS and fresh prepared 0.25 μL each of TAMRA-N_3 (10 mM stock in DMSO, Lumiprobe), $CuSO_4$ (100 mM stock in H_2O), THPTA (Tris(3-hydroxypropyltriazolylmethyl)amine, 10 mM stock in H_2O, Sigma) and sodium ascorbate (100 mM stock in H_2O). The samples were incubated at room temperature for 1 h and the reaction was quenched by boiling in SDS-PAGE loading buffer. 20 μL of each sample was applied to SDS-PAGE and analyzed by Tanon 4600SF fluorescence imaging system.

2.3 Western Blotting

Primary antibodies of Raf-1, Cdk-4, Actin, FASN, LSS, DHCR7, and DHCR24 were ordered from Abclonal. Primary antibodies of HSP70 and CDIPT1 were ordered from Thermo Scientifc. For experiments in Fig. 4D, MCF-7 cells were incubated with DMSO or 2 μM celastrol for 24 and 48 h. For those in Fig. S1D, MCF-7 cells were incubated with DMSO, 1 μM and 5 μM celastrol for 18 h. The cells were washed with PBS, harvested, and lysed with lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.5% Triton X-100, 0.1% SDS and complete protease inhibitors) at 4 °C for 30 min. The lysates were centrifuged at 14000 g for 5 min and the soluble fractions were diluted to 3 mg/mL with lysis buffer. Equal amount of protein samples was resolved by SDS-PAGE and then transferred onto nitrocellulose membrane (Millipore). The membrane was blocked

with 3% (w/v) BSA in tris-buffered saline (TBS) for 2 h at room temperature. After blocking, membranes were incubated with respective primary antibodies at 4 °C overnight. After washing three times with TBST (TBS containing 0.1% Tween-20), blots were further incubated with the HRP-conjugated anti-rabbit (Sangon Biotech) secondary antibody for 1 h at room temperature. After incubation, the blot was washed again with TBST three times and developed by high sensitive ECL luminescence reagent (Sangon Biotech). The results are a representative of three biologically independent experiments, and unprocessed images of immunoblots were provided in Fig. S11.

2.4 Gel-based Cel-Dayne profiling of Living MCF-7 cells

MCF-7 cells were grown in DMEM culture medium until 90% confluence. The medium was removed, and cells were individually incubated with DMSO or Cel-Dayne (10 μM) for 2 h as indicated. For competition experiment, MCF-7 cells were pretreated with excess celastrol (20 μM) for 1 h and followed by treating with Cel-Dayne (10 μM) for 2 h. The medium was aspirated, and cells were washed three times with ice-cold PBS to remove the excessive probe followed by UV irradiation (365 nm, 8 watt) for 10 min on ice. The cells were harvested by scraping the cells in ice-cold lysis buffer (50 mM HEPES pH 8.0, 150 mM NaCl, 0.1 mM EDTA, 0.1% Triton X-100 and complete protease inhibitors). The lysed cells were centrifuged at 14000 g for 5 minutes and the soluble fractions were diluted to 2 mg/mL with lysis buffer. 1% SDS (w/v) was added to each sample and click reaction was performed as below: for each reaction, 20 μL protein sample was added with freshly prepared 0.25 μL each of TAMRA-N_3 (10 mM stock in DMSO, Lumiprobe), $CuSO_4$ (100 mM stock in H_2O), THPTA (Tris(3-hydroxypropyltriazolylmethyl)amine, 10 mM stock in H_2O, Sigma) and sodium ascorbate (100 mM stock in H_2O). The samples were incubated at room temperature for 1 h and the reaction was quenched by boiling in SDS-PAGE loading buffer. 20 μL of each sample was applied to SDS-PAGE and analyzed by Tanon 4600SF fluorescence imaging system.

2.5 Quantitative mass spectrometry-based Cel-Dayne profiling of MCF-7 cells

The proteomics experiments were carried out in biological duplicates. The probe incubation and proteome preparation procedures were same as that of labeling experiments described above. Each of 288 μL of cell lysate was subjected to click reaction by adding 3 μL each of Biotin-N_3 (100 mM stock in DMSO, Biomatrick Inc.), $CuSO_4$ (100 mM stock in H_2O), THPTA (Tris(3-hydroxypropyltriazolylmethyl)amine, 10 mM stock in H_2O) and sodium ascorbate (100 mM stock in H_2O). The samples were incubated at room temperature for 3 h and proteins were precipitated by adding ith CH_3OH (600 μL) / $CHCl_3$ (150 μL) / H_2O (300 μL) sequentially and vortexed for a while. After centrifuge at 14,000 g for 3 min, the protein disk was washed twice with sonication in CH_3OH (500 μL), air-dried and re-dissolved in 200 μL binding buffer (50 mM HEPES pH 8.0, 0.5% SDS) by sonication. 50 μL of streptavidin-sepharose (GE Healthcare) beads were added to each sample and incubated at room temperature with continuous rotation for 1 h. The beads were washed with PBS containing 0.5% SDS (w/v) three times, PBS containing 0.5 M NaCl three times,

4 M Urea in 50 mM triethylammonium bicarbonate (TEAB, Thermo Scientific) twice, and 50 mM TEAB five times. Each wash was performed on a rotator for 15 min. The bound proteins were subjected to on-bead reductive alkylation with 200 μL of 10 mM of tris(2-carboxyethyl)phosphine (TCEP, Thermo Scientific) at 56 °C for 30 min and 200 μL of 55 mM iodoacetamide (Sigma) at 37 °C in dark for another 30 min, followed by wash three times with 50 mM TEAB. Bound proteins were digested with 0.25 μg sequencing grade modified trypsin (Promega) reconstituted in 50 μL of 100 mM TEAB overnight at 37 °C. The digests were labeled with respective TMT-6plex Isobaric Label Reagent (Thermo Scientific) according to the manufacturer's procedures. The labeled peptides were combined, desalted by Pierce C18 spin columns, and evaporated to dryness on a SpeedVac.

2.6 Nano LC-MS/MS analysis

Dried peptides were suspended in 20 μL ddH_2O containing 0.1% formic acid with sonication. 5 μL peptide sample was loaded onto a Thermo C18 PepMap100 precolumn (300 μM × 5 mm) and eluted on a Thermo Acclaim PepMap RSLC analytical column (75 μM × 15 cm). Mobile phase A (0.1% formic acid in H_2O) and mobile phase B (0.1% formic acid in acetonitrile) were used to establish the 80 min gradient comprised of 55 min of 4−30% B, 7 min of 30−50% B, and 5 min of 50−90% B 5 min of 90% B, followed by re-equilibrating at 4% B for 8 min. The flow rate was 0.3 μL/min. Peptides were than analyzed on a Thermo Orbitrap Fusion Lumos proteomic mass spectrometer in a data-dependent manner, with automatic switching between MS and MS/MS scans using a cycle time 3 s. MS spectra were acquired at a resolution of 120,000 with an AGC target value of 4×10^5 ions or a maximum integration time of 50 ms. The scan range was limited from 375 to 1500 *m/z*. Peptide fragmentation was performed via high energy collision dissociation (HCD) with the energy set at 38 NCE. The MS/MS spectra were acquired at a resolution of 50,000 with an AGC target value of 1×10^5 ions or a maximum integration time of 10^5 ms. The fixed first *m/z* was 120, and the isolation window was 0.7 *m/z*.

2.7 Data processing

Protein identification and quantification were performed using Proteome Discoverer 2.1 software (Thermo Scientific). Peptide sequences (and hence protein identity) were determined by matching UniProt protein databases with the acquired fragmentation pattern by SEQUEST HT algorithm. The precursor mass tolerance was set to 10 ppm and fragment ion mass tolerance to 0.02 Da. One missed cleavage site of trypsin was allowed. Carbamidomethyl (C) and TMT-6plex (K and N-terminal) were used as a fixed modification. Oxidation (M) was used as variable modifications. All spectra were searched against protein database using a target false discovery rate (FDR) of 1%. The proteins identified in positive group (Cel-Dayne-treated samples) were additionally filtered by at least one unique peptide in each experimental replicate. Protein ratios were calculated as the median of all peptide hits belonging to a protein. Statistical analysis was performed with Perseus 1.5.1.6. Enrichment ratios (Cel-Dayne *vs* DMSO) and competition ratios (Cel-Dayne *vs*

Cel-Dayne with celastrol) obtained from Proteome Discoverer 2.2 were transformed with $\log_2$-(x), and -$\log_{10}$-(*p*-value) were obtained by a two-sided one sample *t*-test over two biological replicates. Only proteins identified with (1) $\log_2$-enrichment ratio > 2.0; (2) $\log_2$-competition ratio > 1.5; and (3) -$\log_{10}$-(*p*-value) regarding $\log_2$-enrichment ratio > 1.3 were considered statistical significant targets. Data are available via ProteomeXchange with identifier PXD028331.

2.8 Competitive GSTO1 alkylation experiment

Recombinant GSTO1 protein (10 μg, 0.1 μg/μL) dissolved in PBS was incubated with DMSO or celastrol (10 μM) at room temperature for 1 h. Each sample was incubated with 100 μM iodoacetamide at room temperature in dark for another 1 h. Then, the samples were subjected to in solution digestion by adding 0.5 μg trypsin (Promega) and incubated at 37 °C overnight. The digests were labeled with respective TMT-2plex Isobaric Label Reagent (Thermo Scientific) according to the manufacturer's procedures. The labeled peptides were combined, desalted by Pierce C18 spin columns, and evaporated to dryness on a SpeedVac. Dried peptides were suspended in 20 μL ddH_2O containing 0.1% formic acid with sonication and analyzed by Thermo Orbitrap Fusion Lumos proteomic mass spectrometer. Data processing was performed using Proteome Discoverer 2.1 software (Thermo Scientific) and peptide sequences were determined by matching protein database with the acquired fragmentation pattern by SEQUEST HT algorithm. The precursor mass tolerance was set to 10 ppm and fragment ion mass tolerance to 0.02 Da. Two missed cleavage sites of trypsin were allowed. TMT-2plex (K and N-terminal) was used as fixed modification. Carbamidomethyl (C) and Oxidation (M) were used as variable modifications. All spectra were searched against protein sequence of human GSTO1 (UniProtKB ID: P78417) using a target false discovery rate (FDR) of 1%.

2.9 Binding site identification with photoaffinity labeling

Recombinant GSTO1 protein (5 μg, 0.2 μg/μL) dissolved in PBS was incubated with Cel-Dayne (20 μM) at room temperature for 1 h, followed by UV irradiation (365 nm, 8 watt) for 10 min on ice. 75 μL of 50 mM NH_4HCO_3 was added, and the samples were reduced with 20 mM DTT at 56 °C for 15 min and alkylated with 50 mM iodoacetamide at room temperature for 30 min in dark. 1 μL ProteaseMax solution (Promega) was added, and the samples were subjected to in solution digestion with 0.5 μg trypsin (Promega) at 37 °C overnight. The digests were desalted by Zip-tip desalting column (Pierce) and evaporated to dryness on a SpeedVac. The dried peptides were suspended in 20 μL ddH_2O containing 0.1% formic acid with sonication and analyzed by Thermo Orbitrap Fusion Lumos proteomic mass spectrometer. Data processing was performed using Proteome Discoverer 2.1 software (Thermo Scientific) and peptide sequences were determined by matching protein database with the acquired fragmentation pattern by SEQUEST HT algorithm. The precursor mass tolerance was set to 10 ppm and fragment ion mass tolerance to 0.02 Da. Two missed cleavage sites of trypsin were allowed. Cel-Dayne (any amino acids), Carbamidomethyl (C), Oxidation (M), were used as variable modifications. All spectra were

searched against protein sequence of human GSTO1 (UniProtKB ID: P78417) using a target false discovery rate (FDR) of 1%. Manual verification was performed to ensure confident peptide identification.

2.10 Cellular thermal shift assay (CETSA)

MCF-7 cells were incubated with DMSO or celastrol (20 μM) in PBS for 1 h, followed by wash three times with ice-cold PBS. For each group, the cells were suspended in PBS (1.2 mL) and aliquot into 7 PCR tubes (each 150 μL). The tubes were heated individually at various temperatures as indicated for 3 min followed by immediate cooling on ice for another 3 min. Each tube was added with 50 μL PBS supplemented with 1.6% NP-40 and protease inhibitors. The cells were then lysed by snap-freeze (in liquid nitrogen)-thaw (25 °C water bath), transferred onto ice and resuspended by pipetting. This cycle was repeated twice. The cell lysates were centrifuged at 21,000 g for 60 min at 4 °C to pellet the denatured and precipitated proteins. Soluble supernatants were analyzed by gel electrophoresis followed by immuno-blotting using respective antibodies as described above.

2.11 Cellular cholesterol quantification

MCF-7 cells were treated with DMSO or 2 μM celastrol for 24 and 48 h. The cells were harvested and washed three times with ice-cold PBS. Total cellular cholesterol was extracted with methanol/chloroform (v/v, 1:2), and quantified using an Amplex Red cholesterol assay kit (Thermo Scientific) according to the manufacturer's protocol. Three independent biological replicates were performed, and values of total cholesterol concentration were normalized to cell number.

3. Procedures for Chemical Synthesis

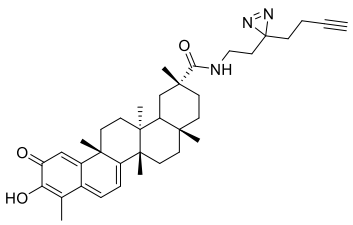
Materials

All reagents were purchased commercially and used without further purification. Anhydrous dimethyl formamide (DMF) were distilled from calcium hydride. Brine refers to a saturated solution of sodium chloride in distilled water. Reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25 mm Huanghai silica gel plates (HSGF254) using UV light as visualizing agent. Flash column chromatography was carried out using Yantai Xinnuo silica.

Celastrol was obtained from MedChemExpress (HY-13067). The “minimalist” diazirine motif 2-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)ethan-1-amine was synthesized as previously described (*Angew. Chem. Int. Ed.* **2013**, *52*, 8551-8556).

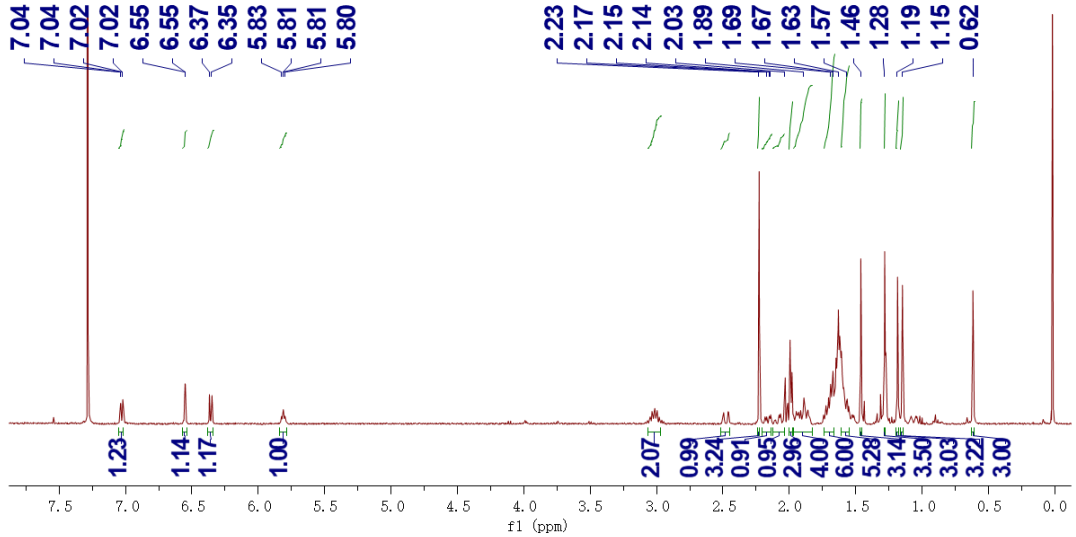
^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker Advance III 500 (^{1}H: 500 MHz, ^{13}C: 125 MHz) with chemical shift values in ppm relative to TMS (δH 0.00 and δC 0.00), residual D-chloroform (δH 7.26 and δC 77.16) as standard. HRMS was obtained using an Agilent 6520 Accurate-Mass Q-TOF instrument.

Cel-Dayne



To a suspension of HATU (11 mg, 0.03 mmol) in anhydrous DMF (0.5 mL) was added a solution of celastrol (10 mg, 0.022 mmol) in anhydrous DMF (1 mL) at 0 °C under a nitrogen atmosphere, followed by the addition of DIPEA (6.5 mg, 0.05 mmol). The reaction mixture was stirred for 15 min, followed by the addition of “minimalist” motif 2-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)ethan-1-amine (4.5 mg, 0.03 mmol) dissolved in anhydrous DMF (0.5 mL). The mixture was continuously stirred for 20 h (0 °C to room temperature). Saturated aqueous NH_4Cl (20 mL) was added to quench the reaction, and the mixture was extracted three times with 20 mL EtOAc. The combined organic layers were washed twice with 10 mL 0.1 M HCl, once with 20 mL saturated $NaHCO_3$, twice with 10 mL brine, and dried over Na_2SO_4. Filtration and solvent removal afforded the crude amide, which was purified by silica gel column chromatography and reverse phase HPLC to get the final product (5.5 mg, 45%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.03(1H, d, *J*=10 Hz), 6.55 (1H, s), 6.36 (1H, d, *J*=10 Hz), 5.81(1H, m), 3.0(1H, m), 2.51(2H, d, *J*=10 Hz), 2.23(3H, s), 2.20-1.75(12H, m), 1.70-1.50(10H, m), 1.46(3H, s), 1.28(3H, s), 1.19(3H, s), 1.15(3H, s), 0.62(3H, s). ^{13}C NMR ($CDCl_3$, 500 MHz) δ 178.2, 177.8, 170.1, 164.7, 146.0, 134.0, 127.4, 119.6, 118.0, 117.0, 82.6, 69.4, 45.0, 44.3, 43.0, 40.4, 39.4, 38.2, 36.4, 34.9, 34.7, 33.7, 33.5, 32.0, 31.6, 31.0, 30.8, 30.2, 29.7, 29.4, 28.7, 27.0, 21.8, 18.4, 13.2, 10.3. HRMS-ESI calcd. for $C_{36}H_{48}N_3O_3$ $[M+H]^+$: 570.3690; Found: 570.3580.

^{1}H-NMR:



^{13}C-NMR: