Electronic Supplementary Material (ESI) for Chemical Science.

< Supplementary Information >

Inflachromene ameliorates Parkinson's disease by targeting Nrf2-binding Keap1

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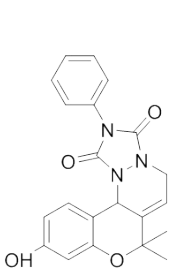
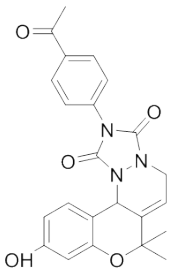
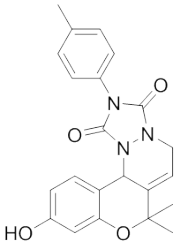
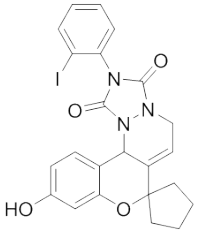
Contents

Supplementary Figures

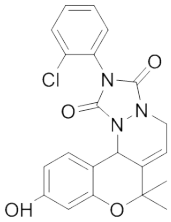


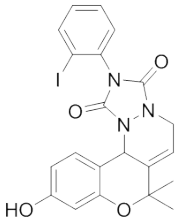


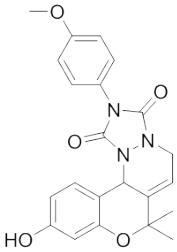


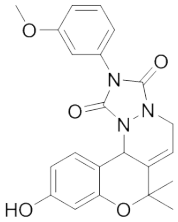


Rank	Top1	Top6	Top7	Top10
Code	ICM	P41G03	P24A09	P24B10
Relative Cell Viability (%)	32.8	25.2	23.5	22.34









Rank	Top11	Top13	Top14	Top15
Code	P24C03	P24A10	P24A07	P24A08
Relative Cell Viability (%)	22.33	21.5	21.0	20.7

Fig. S1 Chemical structures and their rank of benzopyran-embedded compounds in Top 15 compounds.

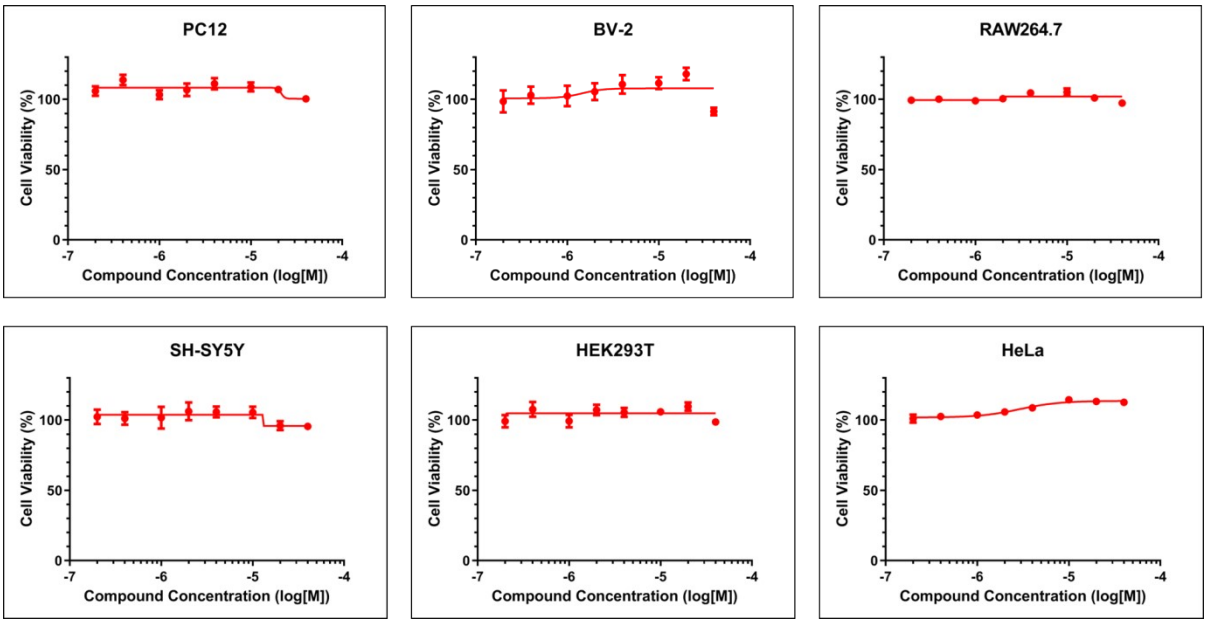


Fig. S2 Cell viability data of ICM with all cell lines used in this study, which confirmed that ICM did not have any cellular toxicity up to 40 µM.

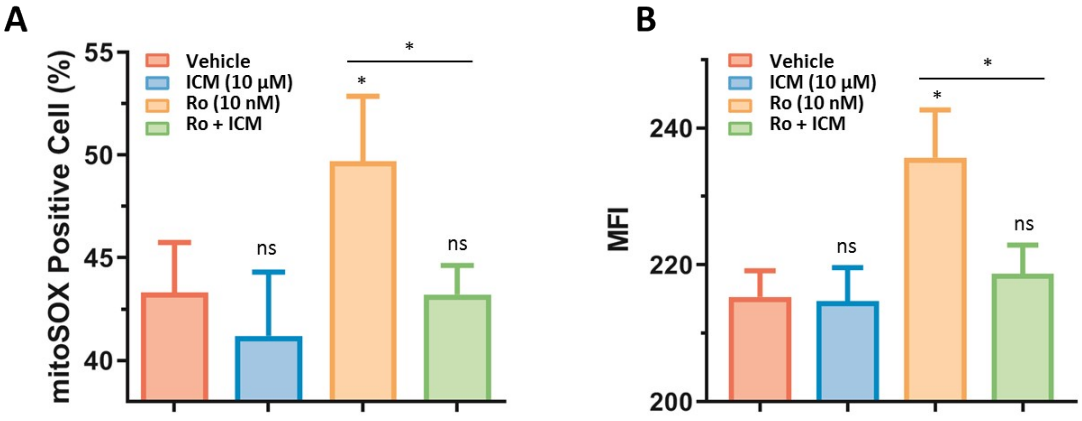


Fig. S3 Flow cytometry data depicting an increase in mitochondrial reactive oxygen species (ROS) upon rotenone treatment, which was counteracted by ICM treatment in PC12 cells (n=3) (Ro; Rotenone) (ns, not significant, $p > 0.05$; *$p < 0.05$).

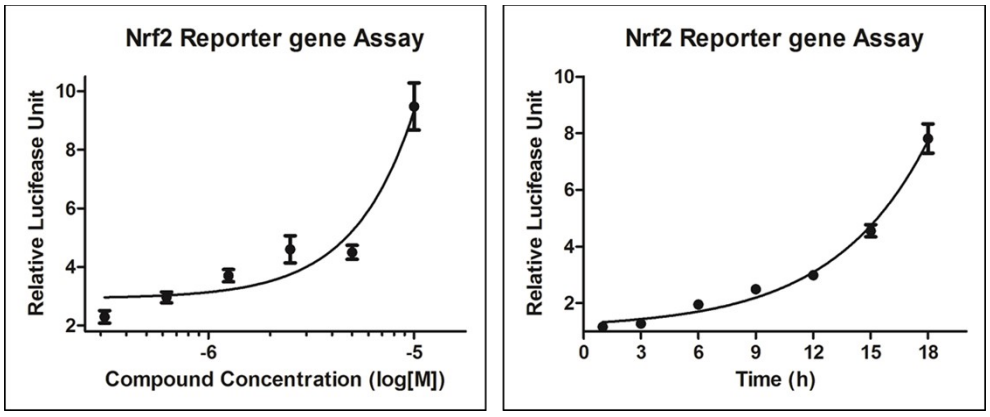


Fig. S4 Nrf2 reporter gene assay showed ICM activated Nrf2 in time- and dose-dependent manners in HEK293T cells (n=6). Data are presented as the mean ± SD.

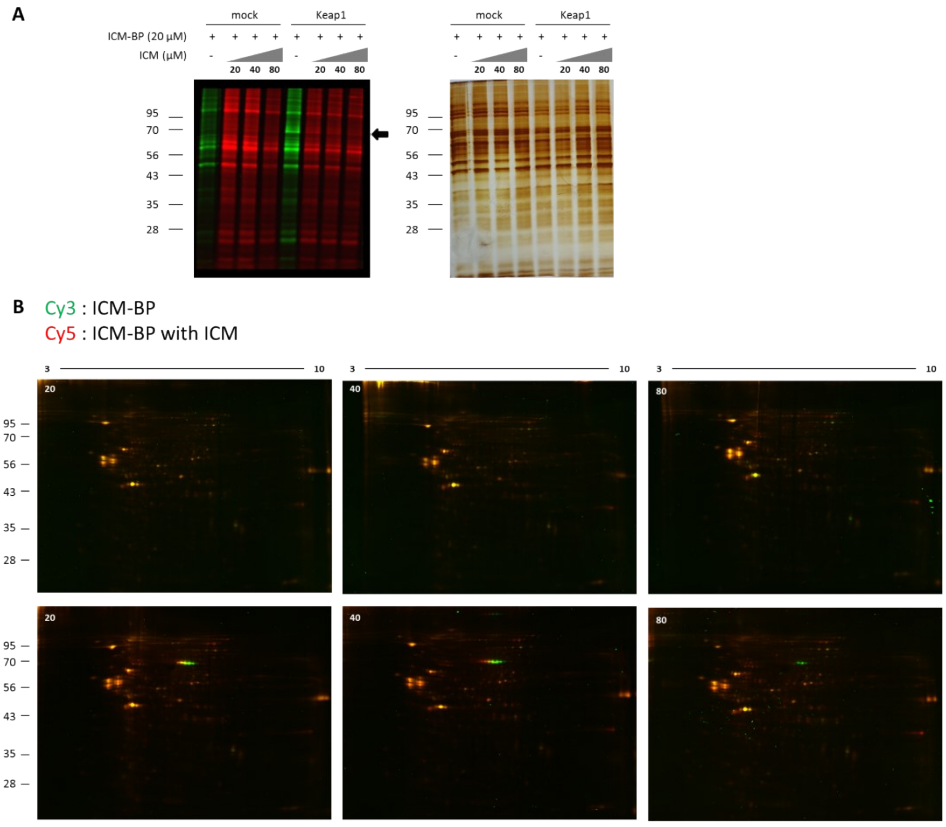


Fig. S5 (A) Silver staining images and (B) 2D gel analysis pertaining to Fig. 2C–D.

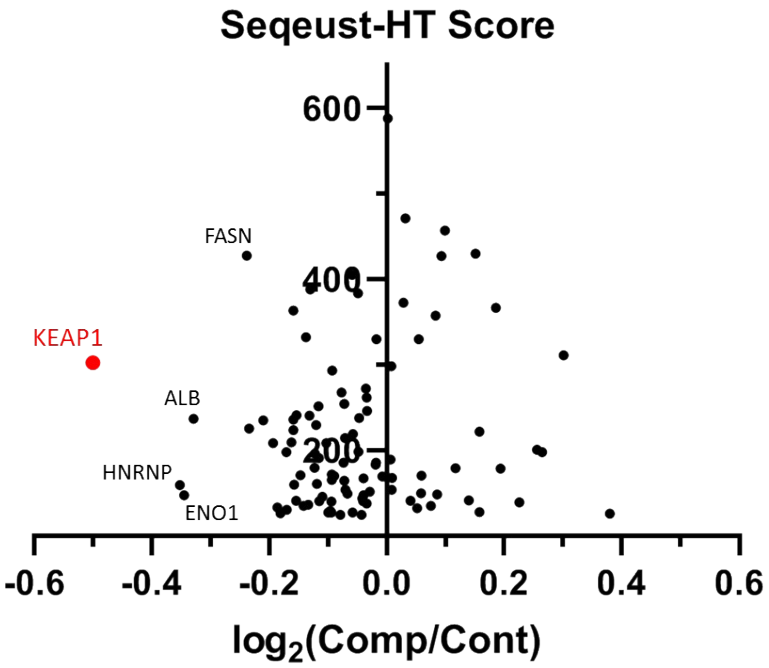


Fig. S6 Chemoproteomics analysis using competition between ICM and ICM-alkyne showed that ICM selectively binds to Keap1. (Cont; ICM-alkyne Control, Comp; Competition between ICM-alkyne and ICM)

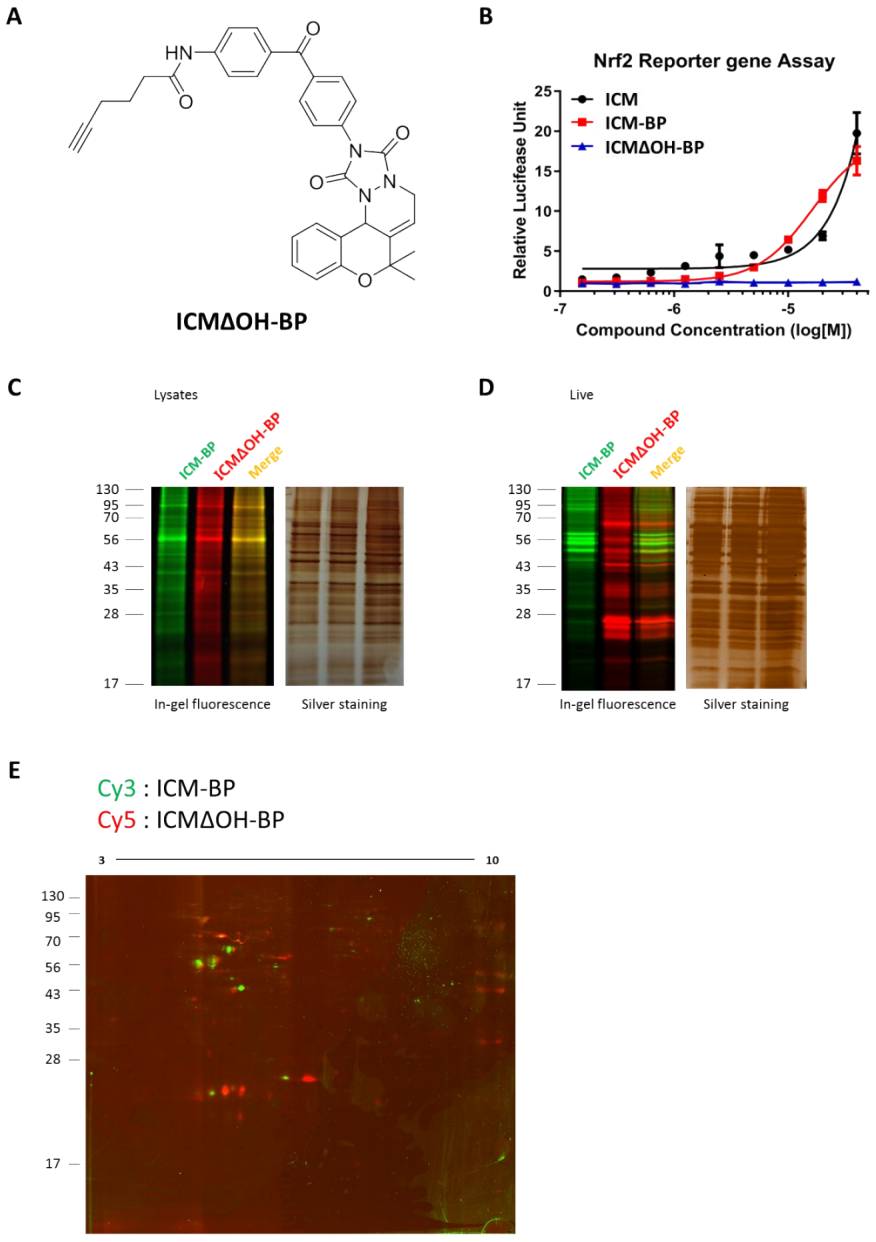
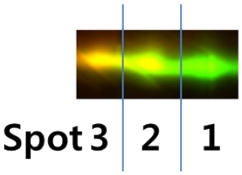


Fig. S7 Target identification of ICMΔOH-BP by 1D and 2D-gel analysis. (A) Chemical structure of target ID negative probe, ICMΔOH-BP. (B) Nrf2 reporter gene assay showed that ICM-BP activated Nrf2 in dose-dependent manners in HEK293T cells, but ICMΔOH-BP did not (n=6). (C–E) ICM-BP and ICMΔOH-BP were labeled by UV irradiation to target proteome in either lysates of HEK293T cells or live HEK293T cells. ICM-BP- and ICMΔOH-BP-labeled proteomes were treated with Cy3-azide (green) and Cy5-azide (red), respectively. Target identification using 1D gel analysis in (C) lysates and (D) live cells. (E) Target identification using 2D gel analysis in live cells. The merged fluorescence images of the whole gel.

A



B

Spot	Gene	Coverage [%]	# PSMs	# Unique Peptides	# AAs	MW [kDa]	calc. pI	Score
1	KEAP1	100.0	2073	77	624	69.6	6.44	7923
2	KEAP1	99.7	1951	76	624	69.6	6.44	7297
3	KEAP1	97.6	1524	72	624	69.6	6.44	5730

Fig. S8 (A) Separated 2D gel spots pertaining to Fig. 2D and (B) their mass spectrometry results pertaining to Table S2.

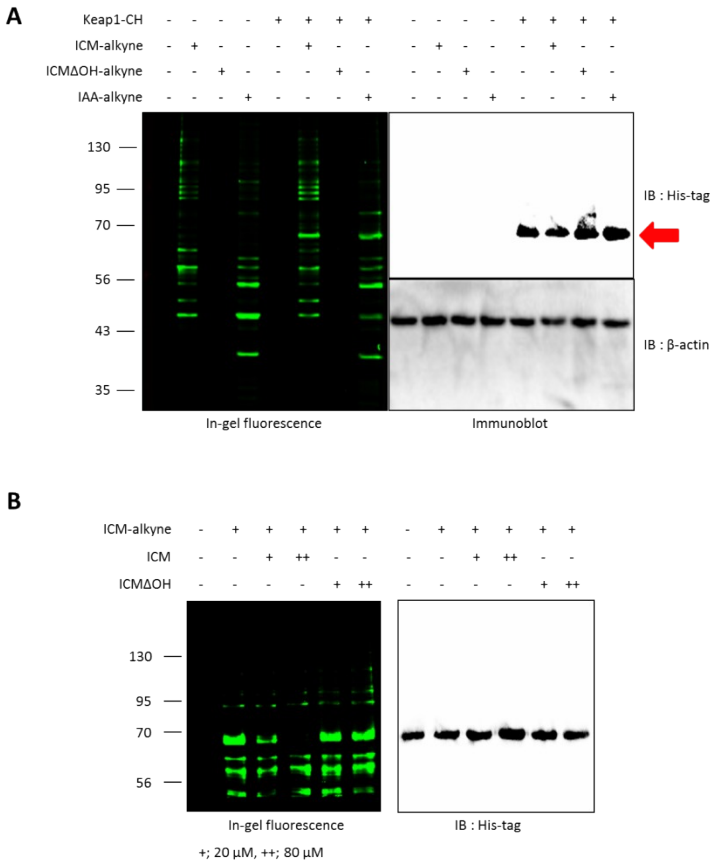


Fig. S9 Uncropped images pertaining to (A) Fig. 4C and (B) Fig. 4D.

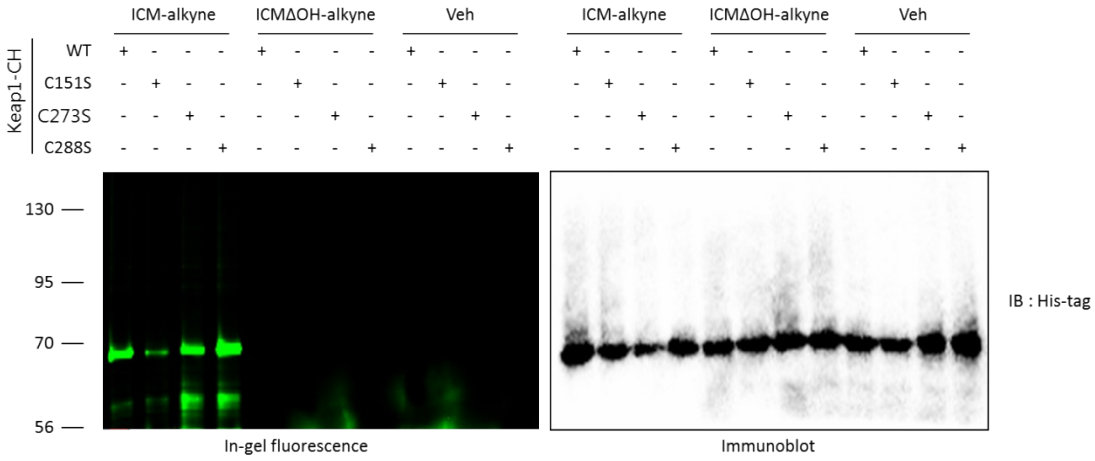


Fig. S10 Uncropped images pertaining to Fig. 5A.

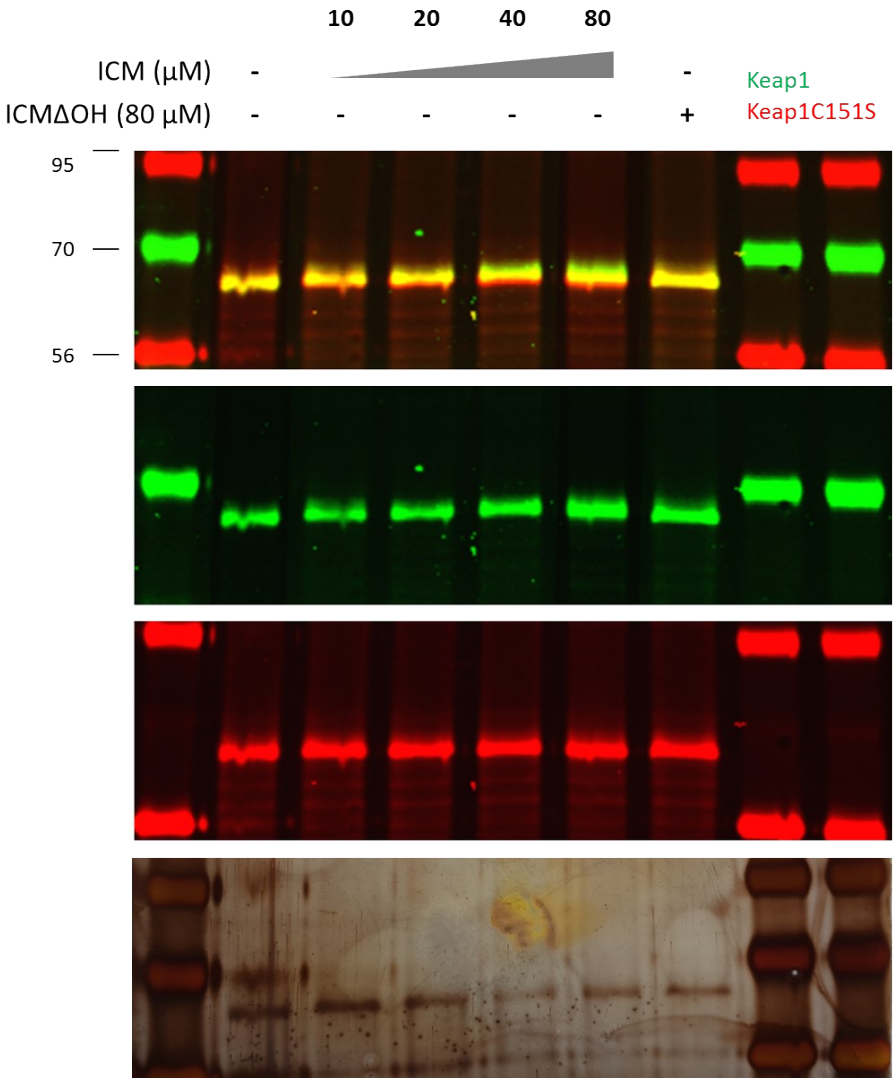


Fig. S11 Uncropped images and silver staining images pertaining to Fig. 5C (green; Keap1, red; Keap1 with C151S point mutation).

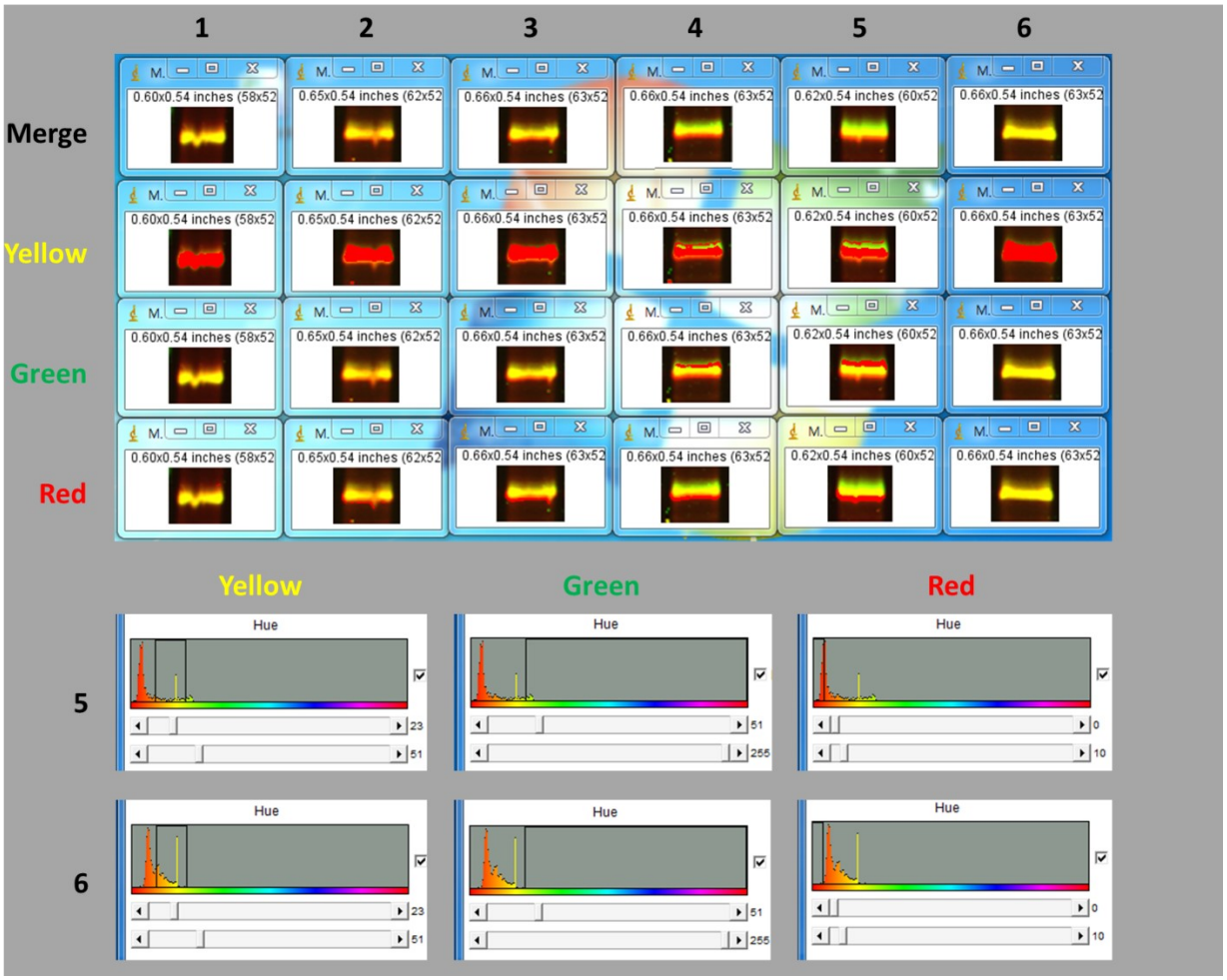


Fig. S12 Quantification method for each color by using ImageJ software pertaining to Fig. 5D. Deep red colored areas were quantified.

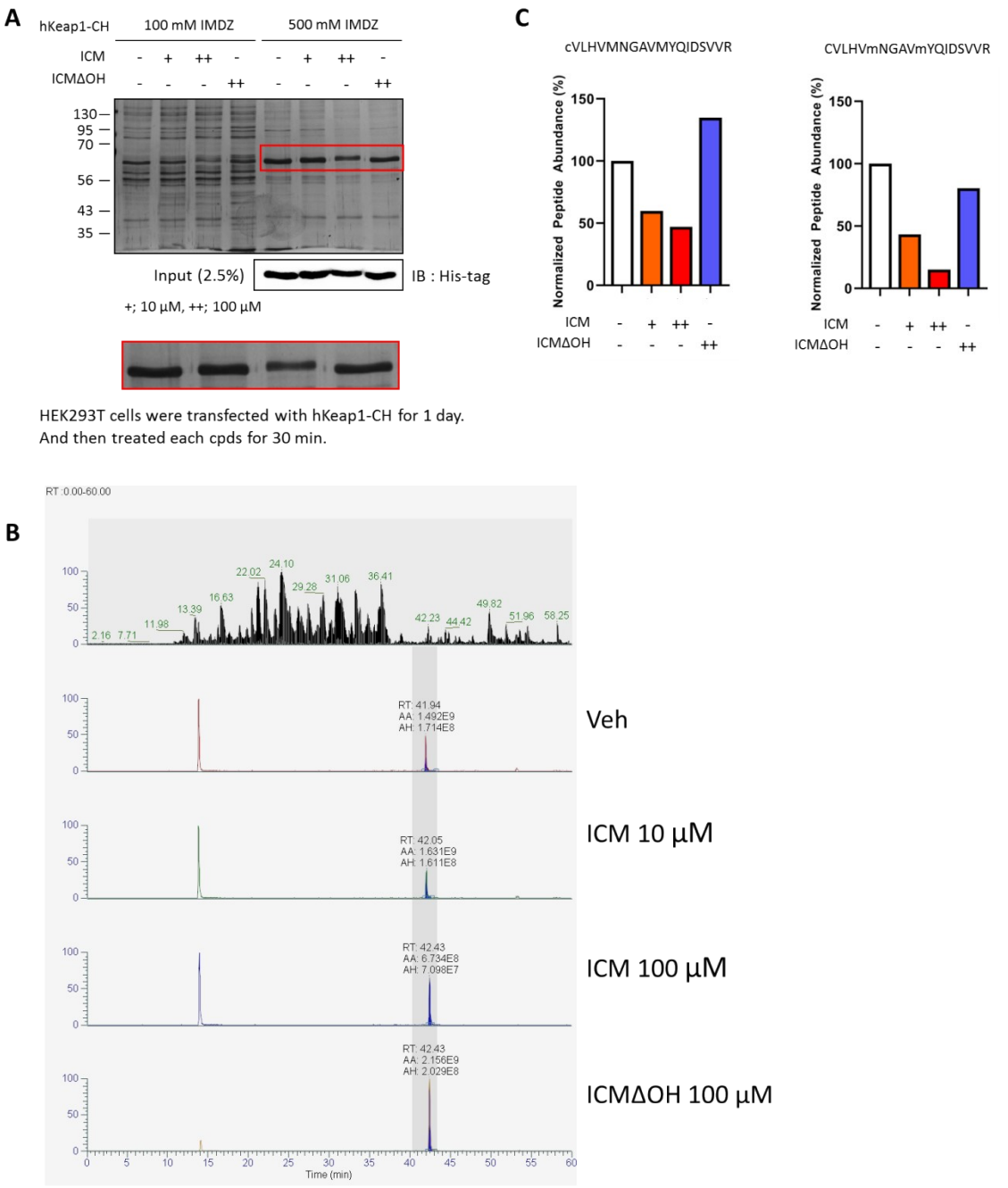


Fig. S13 Mass spectrometry analysis of Keap1 revealed that Cys151 of Keap1 is the major binding site of ICM. (A) Uncropped images pertaining to Fig. 5E. (B) The MS signals of the tryptic peptide pertaining to Fig. 5F. The LC peaks for the peptide around retention time (RT) 42 min is highlighted in gray. (C) The peak areas of the Keap1 tryptic peptide (151–169), which was normalized by Keap1 protein abundance, were decreased upon treatment with ICM, not with ICMΔOH regardless of carbamidomethylation (c; cysteine, C; carbamidomethylated cysteine).

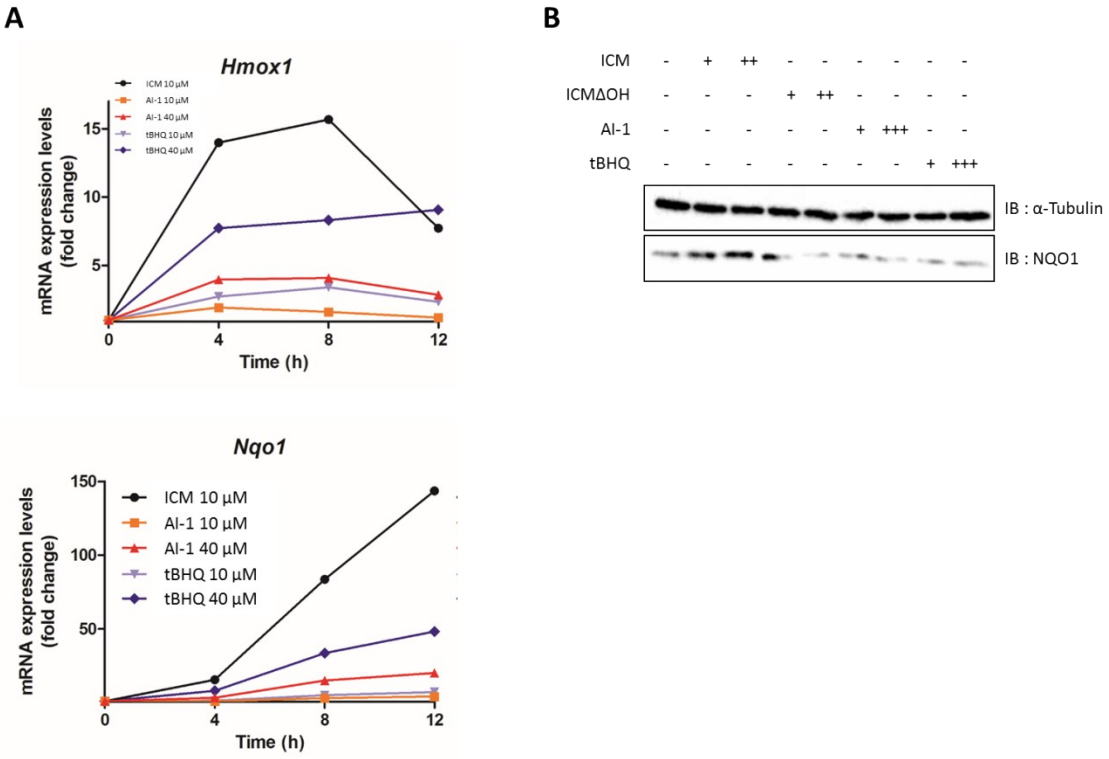


Fig. S14 (A) qPCR analysis of Hmox1 and Nqo1, and (B) immunoblot of NQO1 by ICM, AI-1 and *t*BHQ treatment showed ICM upregulated Hmox1, Nqo1, and NQO1 efficiently in BV-2 cells.

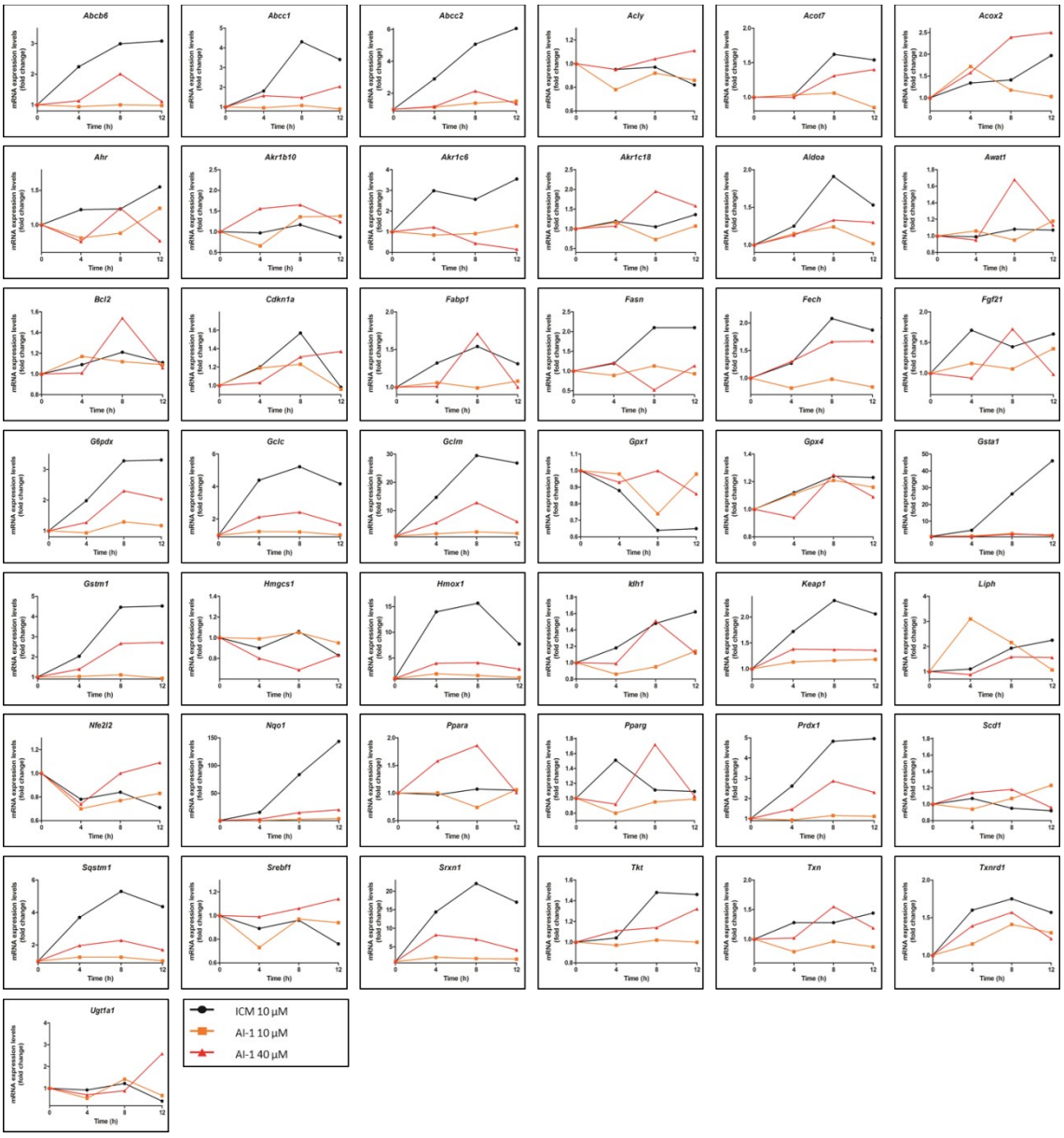


Fig. S15 qPCR analysis of Nrf2-driven 43 genes upon treatment with either ICM (10 μM) or AI-1 (10, 40 μM) after 4, 8, and 12 h.

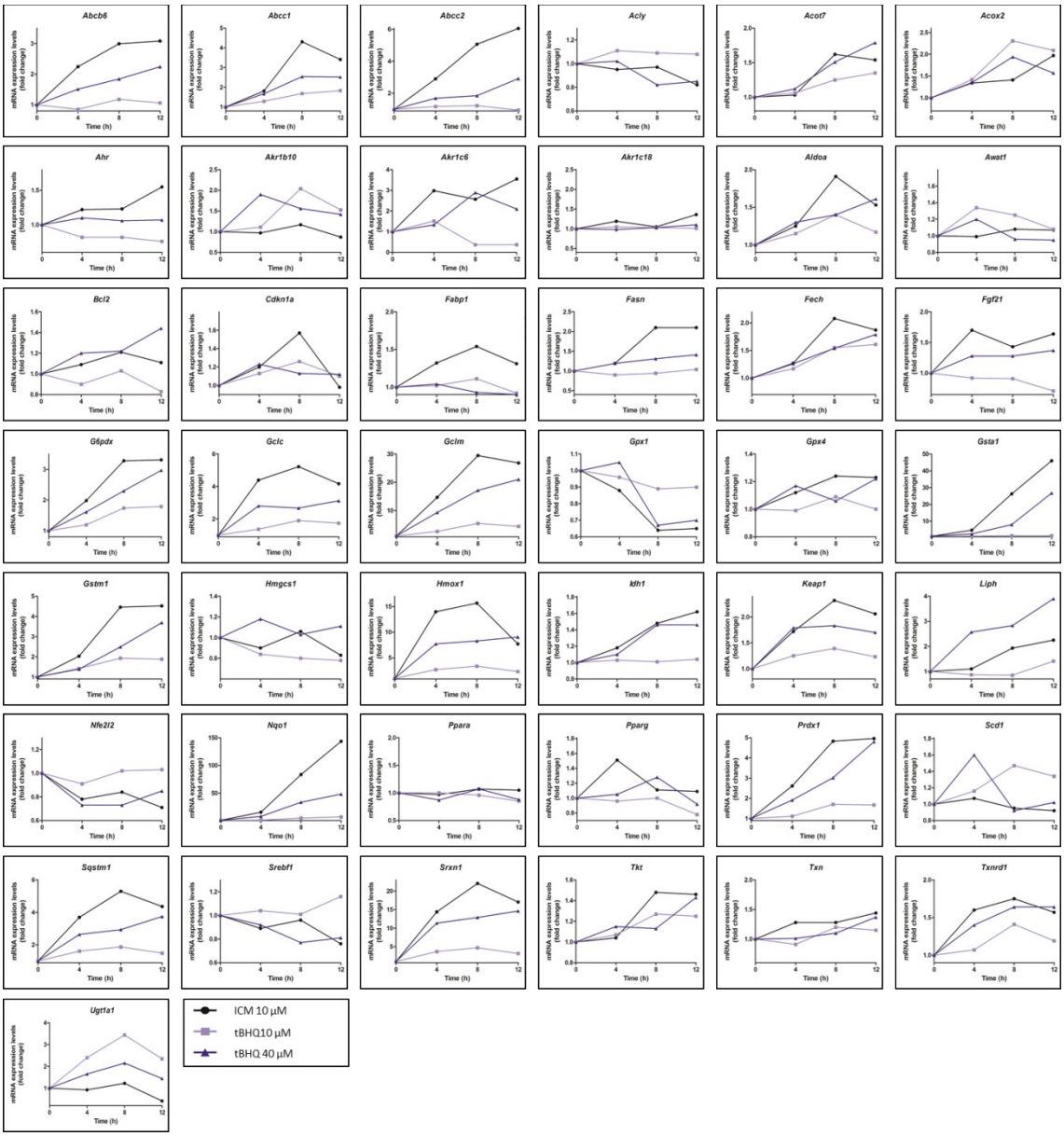


Fig. S16 qPCR analysis of Nrf2-driven 43 genes upon treatment with either ICM (10 μM) or *t*BHQ (10, 40 μM) after 4, 8, and 12 h in BV-2 cells.

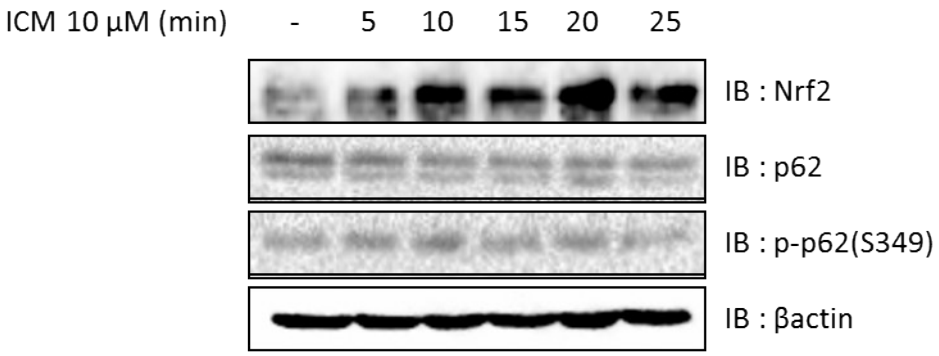
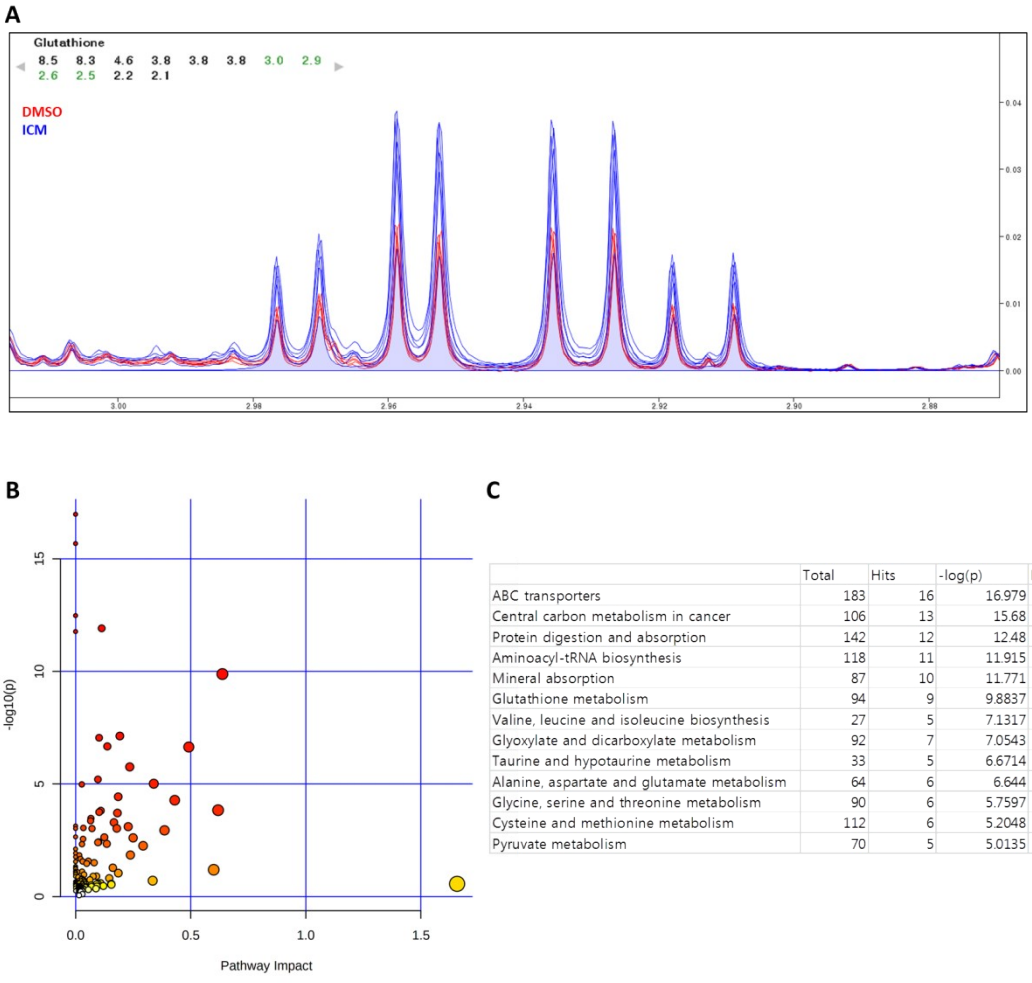


Fig. S17 Immunoblot showed ICM activated Nrf2 within 5 min without any changes in p62 and phosphorylation of Ser349 of p62 in BV-2 cells.



C

	Total	Hits	-log(p)	FDR	Impact
ABC transporters	183	16	16.979	3.47E-15	0
Central carbon metabolism in cancer	106	13	15.68	3.46E-14	0
Protein digestion and absorption	142	12	12.48	3.65E-11	0
Aminoacyl-tRNA biosynthesis	118	11	11.915	1.01E-10	0.1134
Mineral absorption	87	10	11.771	1.12E-10	0
Glutathione metabolism	94	9	9.8837	7.21E-09	0.63768
Valine, leucine and isoleucine biosynthesis	27	5	7.1317	3.49E-06	0.19231
Glyoxylate and dicarboxylate metabolism	92	7	7.0543	3.65E-06	0.10227
Taurine and hypotaurine metabolism	33	5	6.6714	7.51E-06	0.13793
Alanine, aspartate and glutamate metabolism	64	6	6.644	7.51E-06	0.4918
Glycine, serine and threonine metabolism	90	6	5.7597	5.23E-05	0.23529
Cysteine and methionine metabolism	112	6	5.2048	0.000172	0.097087
Pyruvate metabolism	70	5	5.0135	0.000246	0.33962

Fig. S18 (A) Spectra of glutathione upon Vehicle or ICM treatment in BV-2 cells (n=5). (B) Integrated analysis of transcriptome and metabolome data. (C) Pathways pertaining to (B).

Supplementary Tables

Table S1. Mass spectrometry results of the spots in the region of interest pertaining to Fig. 2D.

Spot No.	Match to	Molecular Weight	Mascot Score	Queries matched	Sequence Coverage (%)	Protein
mock-80	SYK_HUMAN	68005	310	39	42	Lysyl-tRNA synthetase
Keap1-80	SYK_HUMAN	68005	264	45	39	Lysyl-tRNA synthetase
	KEAP1_HUMAN	69620	115	12	22	Kelch-like ECH-associated protein 1
	SRP68_HUMAN	70686	115	24	26	Signal recognition particle 68 kDa protein

Table S2. Post-translational modification (PTM) analysis of Keap1 in the three spots in Fig. 3B with mass spectrometry data. Oxidation hindered the binding of ICM to cysteine of Keap1 (Tangerine +; MS/MS validated, Orange-yellow +; MS/MS interference, White blank; not detected).

Position	Target	Modification	Sequence Motif	Spot 3	Spot 2	Spot 1
84	K	Acetyl	DVTLQVkYQDAPA	+	+	+
97	K	Acetyl	AQFMAHkVVLASS	+	+	+
108	K	Acetyl	SSSPVFkAMFTNG		+	+
131	K	Acetyl	IEGIHPkVMERLI	+	+	+
298	K	Acetyl	QSDSRCkDYLVKI	+		
303	K	Acetyl	CKDYLVkIFEELT			+
312	K	Acetyl	EELTLHkPTQVMP	+	+	
551	K	Acetyl	TFVAPMkHRRSAL	+		+
21	S	Phospho	RFLPLQsQCPEGA	+	+	+
33	Y	Phospho	AGDAVMyASTECK	+		
43	T	Phospho	ECKAEVtPSQHGN		+	+
51	T	Phospho	SQHGNRtFSYTLE	+	+	+
53	S	Phospho	HGNRTFsYTLEDH			+
73	S	Phospho	MNELRLsQQLCDV	+	+	+
85	Y	Phospho	VTLQVKyQDAPAA	+	+	+
141	Y	Phospho	RLIEFAyTASISM	+	+	+
162	Y	Phospho	MNGAVMyQIDSVV	+	+	+
182	S	Phospho	VQQLDPsNAIGIA	+	+	+
206	Y	Phospho	HQRAREyIYMHFG	+	+	+
208	Y	Phospho	RAREYIyMHFGEV	+	+	+
224	S	Phospho	EEFFNLsHCQLVT	+	+	+
243	S	Phospho	LNVRCEsEVFHAC	+	+	+
275	S	Phospho	RAVRCHsLTPNFL	+	+	+
277	T	Phospho	VRCHSLtPNFLQM	+	+	+
309	T	Phospho	KIFEELtLHKPTQ	+	+	+
338	S	Phospho	GGYFRQsLSYLEA	+	+	
340	S	Phospho	YFRQSLsYLEAYN	+	+	
341	Y	Phospho	FRQSLSyLEAYNP		+	
345	Y	Phospho	LSYLEAyNPSDGT	+	+	
363	S	Phospho	DLQVPRsGLAGCV	+	+	+
486	S	Phospho	GTNRLNsAECYYP	+	+	+
520	Y	Phospho	VLHNCIyAAGGYD	+	+	+
525	Y	Phospho	IYAAGGyDGQDQL	+	+	+
537	Y	Phospho	LNSVERyDVETET			
541	T	Phospho	ERYDVEtETWTFV	+	+	+
543	T	Phospho	YDVETEtWTFVAP	+		
555	S	Phospho	PMKHRRsALGITV	+	+	+
506	T	Phospho	RSALGItVHQGRI		+	+
576	T	Phospho	GGYDGHtFLDSVE			+
580	S	Phospho	GHTFLDsVECYDP	+		+
602	S	Phospho	RMTSGRsGVGVAV	+	+	+
23	C	Sulfonylation	LPLQSQcPEGAGD	+	+	+
77	C	Sulfonylation	RLSQQLcDVTLQV	+		
151	C	Sulfonylation	ISMGEKcVLHVMN	+	+	
241	C	Sulfonylation	DDLNVRcESEVFH	+	+	
288	C	Sulfonylation	QMQLQKcEILQSD	+	+	
297	C	Sulfonylation	LQSDSRcKDYLVK	+	+	
319	C	Sulfonylation	PTQVMPcRAPKVG	+		

Table S3. Mass spectrometry data of tryptic Keap1 protein upon treatment with either ICM or ICMΔOH pertaining to Fig. 5G–F.

Annotated Sequence	Modificati	Positions in Maste	Theo. MH	Abundanc	Abundanc	Abundanc	Abundanc	Abundanc	Abundanc	Abundanc	Abundanc	Abundanc	Abundanc	Abundanc	Abundanc	Abundanc	Abundanc	Quan Info	Found in S	Found in S	Found in S	Found in S	Confidenc	XCorr (by	Species N	Top Apex
[K].CVLHVMNGAVMY	1xCarbam	Q14145 [151-169]	2191.082	1.348	0.599	0.468	0.679747	0.478225	0.66687	0	0	0	1.00	0.60	0.47	1.35		High	High	High	High	High	6.85	Homo sap	42.31
[K].CVLHVMNGAVMY	2xOxidati	Q14145 [151-169]	2166.05	0.804	0.432	0.149	0.943297	0.316521	0.219246	0	0	0	1.00	0.43	0.15	0.80		High	High	High	Peak Four	High	2.77	Homo sap	50.74
[R].ACSDFLVQQLDPS	2xCarbam	Q14145 [170-202]	3700.785	1.147	0.561	0.186	0.789994	0.399148	0.14366	0	0	0	1.00	0.56	0.19	1.15		High	High	High	High	High	9.63	Homo sap	58.51
[R].ACSDFLVQQLDPS	2xCarbam	Q14145 [170-204]	3927.923	0.64	0.588	0.01	0.977042	0.456096	3.23E-17	0	0		1.00	0.59		0.64		High	High	High	Not Found	High	6.81	Homo sap	55.81
[R].AREYIYMHFGEVAI	1xCarbam	Q14145 [203-234]	3915.931	2.529	0.144	0.01	0.217624	0.000385	3.23E-17	0	0		1.00	0.14		2.53		High	High	High	Not Found	High	11.2	Homo sap	46.9
[R].EYIYMHFGEVAKQ	1xCarbam	Q14145 [205-234]	3688.793	1.906	0.207	0.01	0.420992	0.004781	3.23E-17	0	0		1.00	0.21		1.91		High	High	High	Not Found	High	9.15	Homo sap	50.12
[K].QEEFFNLSHCQLV	1xCarbam	Q14145 [217-234]	2222.091	0.751	0.646	0.286	0.982697	0.601406	0.337025	0	0	0	1.00	0.65	0.29	0.75		High	High	High	High	High	6.25	Homo sap	49.3
[K].QEEFFNLSHCQLV	1xCarbam	Q14145 [217-240]	2933.457	1.514	0.699	0.294	0.602959	0.645307	0.350877	0	0	0	1.00	0.70	0.29	1.51		High	High	High	High	High	5.89	Homo sap	46.42
[R].LIYTAGGYFRQSLS		Q14145 [327-354]	3241.595	1.591	0.512	0.01	0.572369	0.433836	3.23E-17	0	0		1.00	0.51		1.59		High	High	High	Not Found	High	7.64	Homo sap	54.43
[R].IGVGVIDGHIYAVG	1xCarbam	Q14145 [416-459]	4963.427	0.99	0.618	0.01	0.866529	0.516934	3.23E-17	0	0		1.00	0.62		0.99		High	High	High	Not Found	High	7.14	Homo sap	37.51
[R].IGVGVIDGHIYAVG	1xCarbam	Q14145 [416-459]	4964.411	0.01	0.647	0.01	4.11E-17	0.667331	3.23E-17		0		1.00	0.65				High	Not Found	High	Not Found	High	7.75	Homo sap	38.5
[R].MITAMNTIRSGAG	2xCarbam	Q14145 [499-536]	4142.915	0.749	0.677	0.58	0.999139	0.637837	0.662768	0	0	0	1.00	0.68	0.58	0.75		High	Peak Four	Peak Four	Peak Four	High	5.59	Homo sap	58.27
[R].SGAGVCVLHNCIY	2xCarbam	Q14145 [508-551]	4910.202	0.328	0.029	0.014	0.606229	1.28E-08	4.12E-06	0	0	0	1.00	0.01	0.01	0.33		Peak Four	High	High	Peak Four	High	5.55	Homo sap	54.65
[R].SGAGVCVLHNCIY	2xCarbam	Q14145 [508-551]	4924.229	1.844	0.607	0.01	0.44831	0.590436	3.23E-17	0	0		1.00	0.61		1.84		High	High	High	Not Found	High	8.05	Homo sap	45.8
[R].SALGITVHQGRIYV	1xCarbam	Q14145 [555-596]	4730.21	1.196	0.168	0.01	0.810956	0.003436	3.23E-17	0	0		1.00	0.17		1.20		High	High	Peak Four	Not Found	High	6.22	Homo sap	49.04
[R].SGVGVAVTMEPCF		Q14145 [602-615]	1433.724	0.01	0.188	0.15	4.11E-17	0.010275	0.229697		0	0	1.00	0.19	0.15			High	Not Found	Peak Four	Peak Four	High	3.72	Homo sap	21.85
[K].QAFGIMNELRLSQ	1xCarbam	Q14145 [62-84]	2691.396	1.338	0.581	0.35	0.683787	0.548476	0.5317	0	0	0	1.00	0.58	0.35	1.34		High	High	High	High	High	6.89	Homo sap	49.17
[R].LSQQLCDVTLQVK	1xCarbam	Q14145 [72-108]	4105.125	1.002	0.637	0.01	0.878814	0.623738	3.23E-17	0	0		1.00	0.64		1.00		High	High	High	High	High	8.87	Homo sap	43.84
[K].YQDAPAAQFMAH	1xDeamid	Q14145 [85-116]	3483.755	1.787	0.695	0.01	0.640578	0.656831	3.23E-17	0	0		1.00	0.69		1.79		High	High	Peak Four	Not Found	High	6.83	Homo sap	46.46
[K].VVLASSSPVFKAM	1xDeamid	Q14145 [98-131]	3658.897	1.351	0.587	0.11	0.679747	0.4529	0.03492	0	0	0	1.00	0.59	0.11	1.35		High	High	High	Peak Four	High	8.33	Homo sap	46.89

Biological Experimental Procedures

Cell culture: The murine microglial cell line, BV-2, was obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco; #11995-073), supplemented with 1% (v/v) antibiotic-antimycotic solution (Gibco; #15240-062), and heat-inactivated 5% (v/v) fetal bovine serum (FBS) (Gibco; #16000-044). The human neuroblastoma cell line, SH-SY5Y, was obtained from ATCC and cultured in advanced DMEM (Gibco; #12634-010), supplemented with 1% (v/v) antibiotic-antimycotic solution (Gibco; #15240-062), and heat-inactivated 10% (v/v) FBS (Gibco; #16000-044). The human embryonic kidney cell line, HEK293T, was obtained from ATCC and cultured in DMEM (Gibco; #11995-073), supplemented with 1% (v/v) antibiotic-antimycotic solution (Gibco; #15240-062), and heat-inactivated 10% (v/v) fetal bovine serum (FBS) (Gibco; #16000-044). The rat pheochromocytoma cell line, PC12, was obtained from the Korean Cell Line Bank and cultured in Rosewell Park Memorial Institute (RPMI) medium (Gibco; #11875-119), supplemented with 1% (v/v) antibiotic-antimycotic solution (Gibco; #15240-062), heat-inactivated 10% horse serum (HS) (Gibco; #16050-122), and heat-inactivated 5% (v/v) FBS (Gibco; #16000-044). All cell types were maintained in 100-mm cell culture dishes in a humidified atmosphere of a 5% CO_2 incubator at 37 °C. SH-SY5Y and HEK293T cells were cultured every 2 days using trypsin (Gibco; #12605-010), while BV-2 and PC12 cells were detached by pipetting without using trypsin and cultured every 2 days.

Recovered cell viability: To evaluate the recovered cell viability, PC12 cells were seeded at a density of 60,000 cells per well in a transparent 96-well plate (Corning; #3598). Following a 22-h incubation period, the cells were pre-treated with 10 μM of the compound for 2 h, followed by co-treatment with 100 nM of rotenone. After 24 h of compound and rotenone co-treatment, 10 μl of Ez-cytox solution (Dogen; EZ-BULK150) was added to each well in the 96-well plate. Subsequently, the plate was incubated for an additional 30 min, and the absorbance at 455 nm was measured using a microplate reader (Biotek; Synergy).

Reporter gene assay: For the reporter gene assay, HEK293T cells were seeded at a density of 20,000 cells per well in a white 96-well plate (Falcon; #353296). The cells were then transfected with a 2 to 1 ratio of pARE-luc and pRL-TK (Promega; E2241) mixture using LTX plus (Invitrogen; 15338100) and opti-MEM (Gibco; #31985070), following the manufacturer's protocol. After 24 h of incubation, the culture

medium was replaced with fresh medium, and the cells were treated with either ICM or ICMΔOH for an additional 24 h. To measure the Nrf2 activation, the cells were washed with PBS and lysed with 20 µl of 1× passive lysis buffer for 15 min. Subsequently, luciferase signals were measured using the dual-luciferase reporter assay system (Promega; E1980) with a microplate reader (Biotek; Synergy HTX). The expression level of Nrf2 was normalized using the renilla luciferase signal, allowing us to assess the impact of ICM and ICMΔOH on Nrf2 activation in HEK293T cells.

Flow cytometry analysis: To conduct flow cytometry analysis, SH-SY5Y cells were treated with or without ICM and rotenone for a 24-h duration. After the treatment, the cells were trypsinized and suspended in PBS containing 2% FBS (Gibco; #16000-044). For the measurement of mitochondrial reactive oxygen species (ROS), an equal number of re-suspended cells were stained with a final concentration of 2.5 µM of mitoSOX (Invitrogen; M36008). Subsequently, the stained cells were subjected to flow cytometry analysis using FACSAria II (BD). This allowed us to assess and compare the levels of mitochondrial ROS in cells exposed to different conditions, providing valuable insights into the impact of ICM and rotenone on SH-SY5Y cells.

Immunoblotting: For immunoblotting, cells were harvested and lysed in a modified radioimmunoprecipitation assay (RIPA) buffer [containing 150 mM NaCl, 1% IGEPAL CA-630 (Sigma; I8896), 0.5% deoxycholate (Sigma; #30970), 5 mM NaF (Sigma; S7920), 2 mM Na3VO4 (Sigma; #450243), 1× PIC (Roche; #11873580001), and 50 mM Tris–HCl, pH 7.8] for 20 min on ice. The cell lysates were then clarified by centrifugation at 20,000 g, 4 °C for 20 min. The concentration of the soluble protein fraction was determined using the BCA protein assay kit (Thermo; #23225).

Equal amounts of the protein samples were mixed with 5× SDS sample buffer (Biosesang; SF2002-110-00) and heated at 95 °C for 5 min. Subsequently, the protein samples were separated by SDS-PAGE and transferred to PVDF membranes (Bio-Rad; BR162-0177). The membranes were blocked with 2% bovine serum albumin (BSA, MP Biomedicals; #0216006980) in Tris-buffered saline containing 0.1% Tween-20 (TBS-T, Sigma; P9416) at room temperature for 1 h. To detect the target proteins, the membranes were then incubated overnight at 4 °C with primary antibodies at the following dilutions: Nrf2 (Sigma; #12721S) and HO-1 (Abcam; ab13248) at 1:1000, NQO1 (Abcam; ab34173), p62 (CST; #5114S), p-p62 (phospho S349, CST; #95697S), keap1 (Origene, TA502059), and α-tubulin (CST; #3873S)

at 1:1000, and GAPDH (CST; #2118) and β-actin (CST, #4970) at 1:2000 dilutions. After washing with TBS-T, the membranes were incubated with HRP-conjugated secondary antibodies at room temperature for 1 h. The secondary antibodies used were anti-rabbit (CST; #7074) and anti-mouse (CST; #7076) at a dilution of 1:5000. Finally, chemiluminescent signals from the target proteins were detected using the ECL prime kit (Cytiva; RPN2232) and visualized with ChemiDoc (Bio-Rad). This immunoblotting analysis enabled the detection and quantification of specific proteins, allowing us to study the expression levels and modifications of Nrf2, HO-1, NQO1, p62, p-p62, keap1, α-tubulin, GAPDH, and β-actin in the cell lysates.

Subcellular fractionation: Subcellular fractions of cells, including the nucleus and cytosol, were extracted using the NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo; 78835) following the manufacturer's protocol. Briefly, cells were re-suspended in CER I buffer, vortexed for 15 sec, and then incubated for 10 min at 4 °C. Next, CER II buffer was added to the cell lysates, vortexed for 5 sec, and incubated for an additional 1 min. The mixture was then centrifuged at 13,000 g, 4 °C for 5 min, and the resulting supernatant, containing the cytosolic fraction, was immediately transferred to a clean, pre-chilled e-tube. The insoluble pellet fraction, enriched with nuclei, was re-suspended in NER buffer and incubated for 10 min at 4 °C followed by 15 sec of vortexing. After four cycles of incubation and vortexing, the sample was centrifuged at 13,000 g, 4 °C for 10 min, yielding a clear nuclear fraction that was transferred to another clean, pre-chilled e-tube. The protein concentrations of both the nuclear and cytosolic fractions were determined using the BCA protein assay kit (Thermo; #23225). This subcellular fractionation procedure allowed for the separation and isolation of distinct cellular compartments, facilitating further analysis of nuclear and cytosolic proteins.

Quantitative PCR: Cell lysis was performed using RLT buffer (Qiagen), and RNA extraction was carried out using the RNAeasy PLUS Mini Kit (Qiagen; #74136). The obtained RNA was used to generate cDNA using the AccuPower CycleScript RT PreMix (dT20) kit (Bioneer; K2044) on a C1000 Touch Thermal Cycler (Bio-Rad; #1841000). Subsequently, quantitative PCR (qPCR) was conducted with the KAPA SYBR® FAST qPCR Master Mix (2×) Universal Kit (Kapa; KK4605) on a StepOne Real-Time PCR system (Applied Biosystems; #4376357). To determine the relative expression levels of target genes, the cycling threshold value (CT) of the endogenous control gene, β-actin, was subtracted from the CT value of each

target gene, resulting in the calculation of the shift in cycling threshold (ΔCT). The relative expression of each target gene was then denoted as the "fold change" relative to that of vehicle-treated samples (2-ΔCT). Detailed primer sequences for this qPCR analysis are provided in the table below.

List of qPCR primers

Il6	F: 5’- TCC AGT TGC CTT CTT GGG AC -3’ R: 5’- GTA CTC CAG AAG ACC AGA GG -3’
Il1b	F: 5’- AAG TTG ACG GAC CCC AAA AGA T -3’ R: 5’- TGT TGA TGT GCT GCT GCG A -3’
Il10	F: 5’- ATA ACT GCA CCC ACT TCC CA -3’ R: 5’- GGG CAT CAC TTC TAC CAG GT -3’
Nos2	F: 5’- GCC ACC AAC AAT GGC AAC A -3’ R: 5’- CGT ACC GGA TGA GCT GTG AAT T -3’
Tnf	F: 5’- ATG GCC TCC CTC TCA TCA GTT C -3’ R: 5’- TTG GTG GTT TGC TAC GAC GTG -3’
Ccl2	F: 5’-TCA GCC AGA TGC AGT TAA CG-3’ R: 5’-GAT CCT CTT GTA GCT CTC CAG C-3’
Cxcl10	F: 5’-AAG TGC TGC CGT CAT TTT CT-3’ R: 5’-GTG GCA ATG ATC TCA ACA CG-3’
Gapdh	F: 5’- TGG GCT ACA CTG AGC ACC AG -3’ R: 5’- GGG TGT CGC TGT TGA AGT CA -3’
Abcb6	F: 5'-GCG TTT ATG CCG AGA TGT GG-3' R: 5'-TGC AGT ACC CCA CAC AAC C-3'
Abcc1	F: 5'-AGC AGC CTG TAC GGA TTG TGT-3' R: 5'-CCA ACT GGG AAC TTC CCT TAG G-3'
Abcc2	F: 5'-TGC CCT GGA AAT CAC GAT GG-3' R: 5'-TGC CCT GGA AAT CAC GAT GG-3'
Acly	F: 5'-CTG TGC CAC CAT GTT CTC CTC-3' R: 5'-AGG CCT GGT TCT TGG CTA CTG-3'
Acot7	F: 5'-GGG AGG CAG GTA GCC TTT TC-3' R: 5'-ATT GGC ATC ATC TGG ACG CA-3'
Acox2	F: 5'-AAC GAC ACT GGC AGG AAA CA-3' R: 5'-CAC CGG GAG GTA CCA AGA AC-3'
Ahr	F: 5'-CAC AGA GAC CGG CTG AAC AC-3’ R: 5'-ACT GGT CCT GGC CTC CAT TT-3'
Akr1b10	F: 5'-GAG AGC AGG ACG TGA GAC TTC TAC C-3' R: 5'-CCG ATA TCC TGC ATC AAT GGC CAC C-3'
Akr1c6	F: 5'-CAC ATA TTA CAA CTT ATC CTG AGC C-3' R: 5'-TCA ATC CCT ACA CAC ACA TTT CA-3'
Akr1c18	F: 5'-CTG GTT GCA CAC AGG CTT GTA C-3' R: 5'-CAC CCA TCG TTT GTC TCG TTG AG-3'
Aldoa	F: 5'-CGC GTT CGC TCC TTA GTC CT-3' R: 5'-GAC AGG CGG GTC ATG TTG AA-3'
Awat1	F: 5'-AGT GTG CCT AAC ACC ACC AC-3' R: 5'-ACG TGA AGG TAG GGA CCA GA-3'
Bcl2	F: 5'-CTG AGT ACC TGA ACC GGC AT-3' R: 5'-GGT ATG CAC CCA GAG TGA TG-3'

Cdkn1a	F: 5'-ATT GGA GTC AGG CGC AGA T-3' R: 5'-AAC AGG TCG GAC ATC ACC AG-3'
Fabp1	F: 5'-GGA AGG ACA TCA AGG GGG TG-3' R: 5'-TCA CCT TCC AGC TTG ACG AC-3'
Fasn	F: 5'-GGA GGT GGT GAT AGC CGG TAT-3' R: 5'-TGG GTA ATC CAT AGA GCC CAG-3'
Fech	F: 5'-GGA AAT GCT TTC GGC CAG C-3' R: 5'-TTG GGG TTT GGT GGT CTT GG-3'
Fgf21	F: 5'-CGT CTG CCT CAG AAG GAC TC-3' R: 5'-AAT CCT GCT TGG TCT TGG GG-3'
G6pdx	F: 5'-GCT TGG ACC GCC ATT TTG TC-3' R: 5'-GGC TGG AAG GGA GGT GAT TC-3'
Gclc	F: 5'-ATG TGG ACA CCC GAT GCA GTA TT-3' R: 5'-GTC TTG CTT GTA GTC AGG ATG GTT T-3'
Gclm	F: 5'-CGT GAA GAG CAG GGG AAT CA-3' R: 5'-AGC TGG AGT TAA GAG CCC CT-3'
Gpx1	F: 5'-CAG GAG AAT GGC AAG AAT GA-3' R: 5'-GAA GGT AAA GAG CGG GTG AG-3'
Gpx4	F: 5'-GCA GGA GCC AGG AAG TAA TC-3' R: 5'-GGC TGG ACT TTC ATC CAT TT-3'
Gsta1	F: 5'-CGC AGA CCA GAG CCA TTC TC-3' R: 5'-TTG CCC AAT CAT TTC AGT CAG A-3'
Gstm1	F: 5'-GAG GGC CTC AAG AAG ATC TCT G-3' R: 5'-TTA CTC CAG TGG GCC ATC TTT G-3'
Hmgcs1	F: 5'-TCC CCT TTG GCT CTT TCA CC-3' R: 5'-GCC GCC CAA TGC AAT CAT AG-3'
Hmox1	F: 5'-GCC GAG AAT GCT GAG TTC ATG-3' R: 5'-TGG TAC AAG GAA GCC ATC ACC-3'
Idh1	F: 5'-GAG CTC TCT TGG ACC GAC TT-3' R: 5'-TGC ATC TCC ACC ACA GAA CC-3'
Keap1	F: 5'-GAT CGG CTG CAC TGA ACT G-3’ R: 5'-GGA CTC GCA GCG TAC GTT-3’
Liph	F: 5'-CCC TGC AGG CCC TTT ATT CA-3' R: 5'-TAG CCT AGT GCG TCA GTG TC-3'
Nfe2l2	F: 5'-TCC TAT GCG TGA ATC CCA AT-3’ R: 5'-GCG GCT TGA ATG TTT GTC TT-3’
Nqo1	F: 5'-CGC CTG AGC CCA GAT ATT GT-3' R: 5'-GCA CTC TCT CAA ACC AGC CT-3'
Ppara	F: 5'-TGG TGT TCG CAG CTG TTT TG-3' R: 5'-AGA TAC GCC CAA ATG CAC CA-3'
Pparg	F: 5'-GCC TCA GGT CAG AGT CGC-3’ R: 5'-AGA ACG TGA CTT CTC AGC CC-3'
Prdx1	F: 5'-GGA AAC CAG CAA CAG TTG GG-3' R: 5'-CAA CGG GAA GAT CGT TTA TTG TTA-3'
Scd1	F: 5'-CCG GGA GAA TAT CCT GGT TT-3' R: 5'-CAC TGG CAG AGT AGT CGA AGG-3'
Sqstm1	F: 5'-AGA TGC CAG AAT CGG AAG GG-3' R: 5'-GAG AGG GAC TCA ATC AGC CG -3'
Srebf1	F: 5'-GCA TGC CAT GGG CAA GTA C-3' R: 5'-TGT TGC CAT GGA GAT AGC ATC T-3'

Srxn1	F: 5'-CGG TGC ACA ACG TAC CAA T-3' R: 5'-TTG ATC CAG AGG ACG TCG AT-3'
Tkt	F: 5'-AGC CAG ATC AGC AGA AGC TC-3' R: 5'-TCA TTG TGA GGG TTT CGG GG-3'
Txn	F: 5'-GCT TGT CGT GGT GGA CTT CT-3' R: 5'-CCC CCA CCT TTT GAC CCT TT-3'
Txnrd1	F: 5'-GAC ACT CTA CTA AGT GCC CTG C-3' R: 5'-GGA AAC CAG CAA CAG TTG GG-3'
Ugt1a1	F: 5'-TGG GAG GCT GTT AGT GTT CC-3' R: 5'-AAG GCA GTC CGT CCA AGT TC-3'
Actb	F: 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3' R: 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3'

Site-Directed Mutagenesis (SDM) of Keap1 plasmid: Point-mutated plasmids were generated using the Accupower HotStart Pfu PCR premix (Bioneer; K2302), the Keap1 plasmid (Sino Biological; HG11981-CH), and Keap1 SDM primers. The amplification of DNA was confirmed using a 0.8% agarose gel (stained with 10,000× SYBR gel stain solution, run at 135 V for 25 min). Subsequently, DNA was extracted and purified using the PCR purification kit (Qiagen; #28104) following the manufacturer's protocol.

The purified DNA was then subjected to digestion with Dpn I (Takara; #1235A) at 37 °C for 90 min, followed by inactivation at 70 °C for 15 min. The digested DNA was re-purified using the PCR purification kit. Details of the primer sequences utilized in this analysis are provided in the table below.

List of Keap1 SDM primers

C151S	F: 5'- TCCATGGGCGAGAAG **TCT** GTCCTCCACGTCATG-3' R: 5'- CATGACGTGGAGGAC **AGA** CTTCTCGCCCATGGA -3'
C273S	F: 5'- CTGCGGGCCGTGCGC **TCC** CACTCGTTGACGCCG -3' R: 5'- CGGCGTCAACGAGTG **GGA** GCGCACGGCCCGCAG -3'
C288S	F: 5'- ATGCAGCTGCAGAAG **TCC** GAGATCCTGCAGTCC -3' R: 5'- GGACTGCAGGATCTC **GGA** CTTCTGCAGCTGCAT -3'

FITGE & Click Chemistry: The FITGE procedures were conducted following previously described methods.[1] Briefly, HEK293T cells were transiently transfected with either mock or Keap1 for 1 day. Subsequently, the cells were treated with 20 μM of ICM-BP in the presence or absence of 20, 40, or 80 μM of ICM for 30 min, and then photocrosslinked by 365-nm UV irradiation on ice for 30 min. Afterward, the cells were washed with 1× PBS (Welgene; ML008-02) and lysed in RIPA buffer containing 1× PIC (Roche; #11873580001). Next, 100 μg of proteome was labeled with either 40 μM of Cy3-azide (Lumiprobe; #31030) or Cy5-azide (Lumiprobe; #33030), along with an additional 100 μM of tris[(1-

benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) (Sigma; #678937), 1 mM of $CuSO_4$ (Sigma; C8027), 1 mM of tris(2-carboxyethyl)phosphine (TCEP) (Sigma; C4706), and 5% of *t*-BuOH (TCI; B0706) for 1 h. The dye-conjugated proteomes were then precipitated using cold acetone at –20 °C for 20 min, followed by centrifugation at 20,000 g, 4 °C for 7 min. After three acetone washes, the pellets were re-suspended with 50 µl of re-hydration buffer [7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 40 mM DTT (Millipore; #1.11474.0025), and 1% pH 3–10 IPG buffer (Cytiva; #17-6000-87)].

Equal amount of Veh- and ICM-BP-treated samples were mixed and loaded on a pH 3–10, 24-cm Immobiline Drystrip gel (Cytiva; #17-6002-44). The primary dimension was separated using an isoelectric focusing system (Cytiva; Ettan IPGphor 3), and the secondary dimension was separated by a polyacrylamide gel electrophoresis (PAGE) system (Cytiva; Ettan DALTsix). The resulting 2-dimensional (2D) gel was scanned with a fluorescent gel scanner (Azure Biosystems; Sapphire). Fluorescent intensity was quantified by using ImageJ software (LOCI; University of Wisconsin).

In-gel Digestion and Mass Spectrometry: Protein spots from silver-stained gels in Fig. 2D were excised, de-stained, and digested using trypsin.[2] The resulting peptides were subjected to peptide sequencing using nanoAcquity UPLC-ESI-Q-TOF mass spectrometry (Waters; SYNAPT G2-Si HDMS). Peptides were eluted with a linear gradient of 5–40% buffer B [acetonitrile (ACN)/formic acid; 100:0.1 (v/v)] with buffer A [water/formic acid; 100:0.1 (v/v)] over 80 min, and the MS scan cycle consisted of one MS scan followed by MS/MS scans of the 10 most abundant ions in each MS scan.

Subsequently, the resulting MS data were processed using Protein Lynx Global Server (PLGS) 2.3 data processing software [Waters], and the peaklists were generated as .pkl files. These peaklist files were then searched using the Mascot global search engine (version 2.2.0) against the protein sequence database SwissProt (version 51.6, 257964 entries), considering homo sapiens (human) as the taxonomy filter for 2D-PAGE samples. The identification of protein spots allowed for a maximum of 1 missed cleavage, and no fixed modifications were considered. For the identification of protein spots, the following variable modifications were considered: carbamidomethylation of Cys, oxidation of Met, phosphorylation of Ser or Thr, acetylation and formylation of Lys, *N*-terminal pyroglutamylation of Gln and Glu, and acrylamide adduct propionamide of Cys.

Peptides from protein spots in Fig. 3B, S5, 5E, and S10 were resuspended with 0.1% formic acid and injected onto Ultimate 3000 RSLCnano [Thermo Scientific] coupled to Q Exactive Plus MS System [Thermo Scientific]. Peptides were trapped on an Acclaim PepMap 100 column (75 µm x 2 cm, 3 µm,

Thermo Scientific), separated on an EASY-Spray column (75 µm x 15 cm, 2 µm, Thermo Scientific) for a 1-h gradient of 7–28% acetonitrile containing 0.1% formic acid. MS scans were acquired in the Orbitrap at 70,000 resolution with automatic gain control (AGC) target of 3e6 and maximum injection time of 100 ms. The top 10 precursor ions were selected with data-dependent mode, and MS2 spectra (isolation window 1.5 Th, AGC target 1e5, maximum injection time 250 ms) were acquired with normalized collision energy (NCE) of 28 and analyzed at Orbitrap with 17,500 resolution. Proteome Discoverer 2.4 [Thermo Scientific] was used for protein identification and quantification using SwissProt databases including human. For protein spots in Fig. 3B and S5, methionine oxidation, carbamidomethylation, and oxidation of cysteine residues and *N*-terminal acetylation were set as variable modifications. For protein spots in Fig. 5E and S10, methionine oxidation, phosphorylation of serine, lysine, tyrosine residues, acetylation of lysine residues, carbamidomethylation, oxidation and sulfonylation of cysteine residues and *N*-terminal acetylation were set as variable modifications. False discovery rate (FDR) of < 1% was applied.

Sample preparation and acquisition for quantitative proteome analysis: Proteins were reduced and alkylated with 5 mM DTT (Sigma; #43815) and 15 mM IAA (Sigma; I1149), respectively. The SP3 protocol was applied with Sera-Mag Carboxylate SpeedBead (Cytiva; #45152105050250, #65152105050250). Briefly, the proteins were mixed with Sera-Mag Carboxylate SpeedBead at 24 °C for 5 min at 1,000 rpm and washed using 80% ethanol (Merck; #1009832511). Trypsin solution (ThermoFisher; #90057) in 50 mM TEAB (Sigma; #18597) was added and incubated at 37°C for 16 h at 1,000 rpm. For a tandem mass tags (TMT) labeling-based quantitative proteomic analysis, the peptide samples were labeled with TMT 6-plex reagent (ThermoFisher; #90066) according to the manufacturer's instructions. The mixed labeling samples were desalted with Pierce C18 Spin Tips (ThermoFisher; #84850) and dried using GeneVac evaporator.

Subsequently, analysis of TMT labeled peptides was carried out on High-resolution mass spectrometer, Q Exactive HF-X (ThermoFisher) connected with Ultimate 3000 nanoLC system (ThermoFIsher). The samples were dissolved in 0.1% FA (Sigma; #00940) and loaded onto the trap column, PepMap C18 (ThermoFisher; #164535, 3 µm, 2 cm×75 µm). The loaded peptides were separated using an EASY-spray C18 column (ThermoFisher; ES903) employing a gradient of 5 to 25% of ACN (Merck; #1000291000) in 0.1% FA for 2 h at a flow rate of 250 nL/min at 55°C of column temperature. The Q Exactive HF-X was set to acquire data in positive ion mode using data-dependent

acquisition. Each cycle consisted of one full MS scan, followed by a maximum of 20 MS/MS scans. Full MS scans were collected at a resolution of 120,000, 3e6 AGC target, scan range 350-1500 m/z, and 50-ms maximum injection time. All higher-energy collision-induced dissociation (HCD) MS/MS spectra were acquired at a resolution of 30,000, 1e5 AGC target, 54-ms maximum injection time, 0.7 m/z isolation window, 32% collision energy and 45-sec dynamic exclusion.

MS raw files were processed by Proteome Discoverer software (ThermoFisher; version 2.4) and were searched against the UniProtKB human database. The SEQUEST HT search engine was used with the following parameters: fully tryptic specificity; maximum of two missed cleavage; minimum peptide length of 6; static modifications for TMT tags on lysine residues and peptide N termini and carbamidomethylation of cysteine residues; dynamic modifications for oxidation of methionine; precursor mass tolerance of 10 ppm; and fragment mass tolerance of 0.02 Da. For peptide scoring, Percolator was employed with an identification threshold of 1% false discovery rate (FDR). No imputation of missing values was performed.

NMR sample preparation and acquisition for metabolomics: BV-2 cells were treated in the absence or presence of ICM for 24 h. After washing with cold 1× phosphate-buffered saline (PBS) (Welgene; LB001-02), cells were harvested with cold 80% MeOH (Daejung; #5558-4400) and frozen using liquid nitrogen. The frozen cells were then stored at –80 °C until the NMR experiments. To quantify metabolites, 1H NMR-based metabolic profiling was performed using the cell samples, following previously described procedure.

Upon thawing at 4 °C overnight, proteins in the cell lysates were removed using 3K Amicon membrane filters (Merck Millipore; UFC500396) via centrifugation at 18,213 g, 4 °C for 20 min. Next, 330 µL of filtered cell lysates was mixed with 350 µL of 0.2 M deuterated sodium phosphate buffer (pH 7.0). The NMR solvent, deuterated sodium phosphate buffer, was prepared by mixing 0.2 M sodium phosphate monobasic (Sigma) in deuterium oxide and 0.2 M sodium phosphate dibasic (Sigma) in deuterium oxide. The pH of the NMR solvent was then adjusted to 7.0 ± 0.1 for each sample. Subsequently, a total 630 µL of the sample was mixed with 70 µL of deuterium oxide containing 5 mM TSP-d4 (3-(trimethylsilyl) propionic 2,2,3,3-d4 acid sodium salt). Finally, 600 µL of each sample was transferred into a disposable 5-mm NMR tube (Bruker; Z112273).

All NMR samples were handled before or after the NMR measurement on the Sample Jet (Bruker), an automated device. Prior to NMR measurement, samples were maintained at 4 °C in the Sample Jet

and equilibrated at 25 °C for 3 min. All one-dimensional proton (1D 1H) experiments were carried out at 25 °C on a Bruker Avance III HD 800-MHz NMR spectrometer (Bruker) equipped with a Bruker 5-mm CPTCI Z-GRD probe. The NOESY PRESAT pulse sequence with water presaturation was used to acquire 1H NMR spectra, collecting 65,536 data points with 64 transients, a relaxation delay of 2.0 s, a spectral width of 20 ppm, and an acquisition time of 2.0 s. Before Fourier transformation, a 0.3-Hz line broadening function was applied to all spectra.

Spectral processing.: All acquired spectra were processed, including phasing and baseline correction, using Chenomx NMR Suite Processor software (Chenomx Inc., v7.1). Following the spectral processing, metabolites were identified and quantified using Chenomx NMR Suite Profiler software (Chenomx Inc., v7.1). TSP-d4 served as a chemical shift reference with a value of –0.016 ppm. The identification and confirmation of 29 metabolites were carried out utilizing the 800 MHz library of Chenomx, 2D NMR spectra, and spiking experiments. As an example, a representative 1H NMR spectrum of glutathione is presented in Fig. S7A. The concentrations of the 29 identified metabolites were determined by integrating peak areas of the metabolites and comparing them to the areas of the 5 mM TSP peak.

Transcriptomic and Metabolomic Analysis: RNA expression and processed metabolic data were integrated for analysis using MetaboAnalyst web-based server (v5.0). A total of 43 genes and 29 metabolites were considered, and their respective fold changes were utilized for Joint-Pathway Analysis.

MPTP-induced Parkinson's Disease Model: Male C57BL/6 mice, 8 weeks old and weighing 22–25 g, were used for all experiments. Intraperitoneal (IP) injections of ICM (2 or 5 mpk) or vehicle {distilled water containing 5% DMSO and 40% polyethylene glycol 400 (TCI; N0433)} were administered daily for 6 days. On day 1, a quadruple challenge of 20 mpk MPTP in the vehicle was administered with IP injections at 2-h intervals. All mice were sacrificed on day 7 for further analysis. All experiments were performed in accordance with the approved animal protocols and guidelines established by Committee on Animal Research of Kyung Hee University [KHU-ASP-20-235].

Statistics: Statistical analyses of the entire experiments were conducted using Student's *t*-test with

GraphPad Prism software. Data are presented as the mean ± S.D. (standard deviation), as indicated in the Figure legends. The P-value is also indicated in the Figure legends.

General Information for Chemical Synthesis

A. Materials

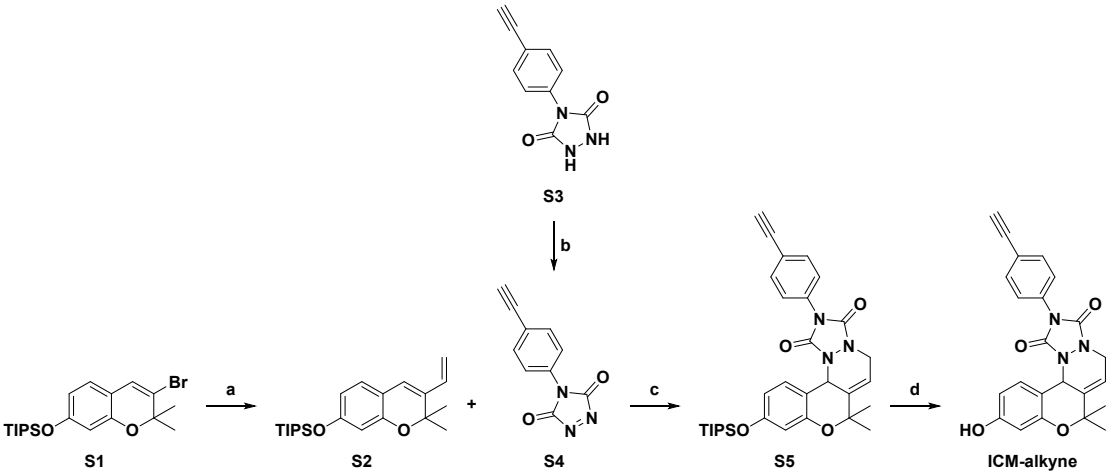
All commercially available reagents for organic synthesis were purchased from Sigma-Aldrich, Tokyo Chemical Industry Co., Ltd, or ThermoFisher Scientific and used without further purification unless otherwise specified. Solvents were purchased from commercial vendors and used without further purification unless otherwise mentioned. Dry solvents were prepared using ultimate solvent purification system CT-SPS-SA [Glass Contour]. The progress of reaction was monitored using thin-layer chromatography (TLC) (silica gel 60, F_{254} 0.25 mm). Components on TLC were visualized by observation under UV light (254 nm) or treating the TLC plates with *p*-anisaldehyde, $KMnO_4$, or phosphomolybdic acid followed by heating. Compounds were purified by flash column chromatography or medium-pressure liquid chromatography (MPLC) Isolera One [Biotage] on silica-gel (230–400 mesh). The eluent used for purification is reported in parentheses. Reverse-phase preparative HPLC was performed with LC-6AD [Shimadzu] using YMC-Pack ODS-A AA20S05-2520WT (S-5 µm, 12 nm, 20 × 250 mm) [YMC]. The eluent and flow rate used for purification is reported in parentheses.

B. Compound characterization

^1H and ^{13}C NMR spectra were obtained on Bruker AVANCE III HD 500 [Bruker]. Chemical shifts were reported in parts per million (δ, ppm). ^1H NMR spectra were calibrated using the residual solvent peak ($CDCl_3$ 7.26 ppm; DMSO-d_6 2.50 ppm) or tetramethylsilane (TMS, 0.00 ppm) as an internal standard. ^{13}C NMR spectra were calibrated using the residual solvent peak ($CDCl_3$ 77.00 ppm; DMSO-d_6 39.52 ppm). Multiplicity was noted as: s (singlet); d (doublet); t (triplet); q (quartet); quin (quintet); m (multiplet); dd (doublet of doublet); dt (doublet of triplet); td (triplet of doublet); ddd (doublet of doublet of doublet); br s (broad singlet), etc. Coupling constants were reported in Hz. Low-resolution mass spectrometry (LRMS) was obtained by LCMS-2020 [Shimadzu] using electron spray ionization (ESI). High-resolution mass spectrometry (HRMS) of final compounds was confirmed by Orbitrap Explorise 120 [ThermoFisher Scientific].

Synthesis and Characterization of Compounds

A. ICM-alkyne



Scheme 1. Synthesis of ICM-alkyne. Reagents and conditions: a) vinylboronic acid dibutyl ester, $Pd(PPh_3)_4$, Na_2CO_3, toluene/EtOH/H_2O (2:2:1, v/v/v), 100 °C, 2 h; b) $PhI(OAc)_2$, THF, r.t., 20 min; c) THF, r.t., 2 h; d) HF/pyridine, THF, r.t., 8 h.

2-(4-ethynylphenyl)-10-hydroxy-7,7-dimethyl-5,12b-dihydro-1*H*,7*H*-chromeno[4,3-*c*][1,2,4]triazolo[1,2-*a*]pyridazine-1,3(2*H*)-dione (ICM-alkyne).

To a solution of **S1**[1)] (182.8 mg, 444.29 µmol, 1 equiv.) in toluene (2 mL) and EtOH (2 mL) at room temperature (r.t.) were added Na_2CO_3 (141.27 mg, 1.33 mmol, 3 equiv.) in H_2O (1 mL), $Pd(PPh_3)_4$ (25.67 mg, 22.21 µmol, 5 mol%) and vinyl boronic acid dibutyl ester (98.14 mg, 533.14 µmol, 117.54 µL, 1.2 equiv.), then stirred at 100 °C for 2 h. After completion of the reaction checked by TLC and LC-MS, the reaction mixture was extracted with ethyl acetate (EtOAc) three times under brine. The combined organic layer was dried over anhydrous Na_2SO_4(s) and concentrated *in vacuo* after filtration. The residue was purified by short silica-gel flash column chromatography (5% EtOAc in hexane) to afford **S2**. This was used in the next step without any further purification.

To a mixture of **S3**[2)] (134.08 mg, 666.45 µmol, 1 equiv.) and $PhI(OAc)_2$ (225.39 mg, 699.77 µmol, 1.05 equiv.) was added tetrahydrofuran (THF, 3 mL), then stirred at r.t. for 20 min. This was used in the next step without any further purification.

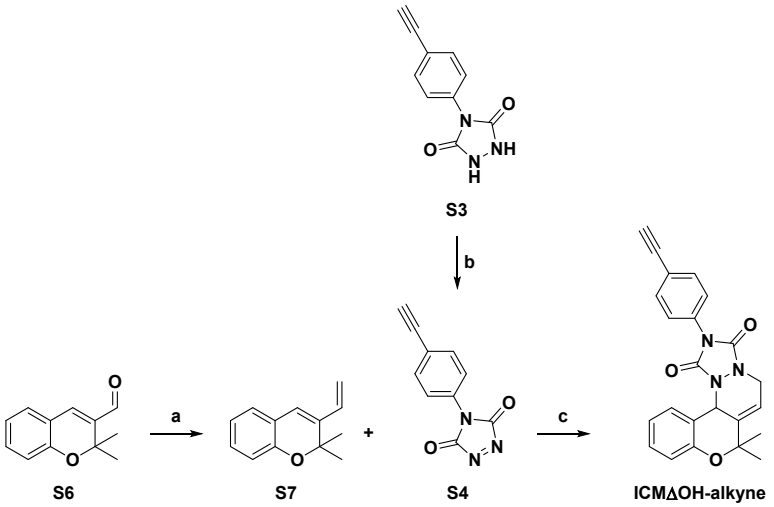
To the reaction mixture of **S4** (132.73 mg, 666.45 µmol, 1.5 equiv.) at r.t. was added **S2** (159.32 mg, 444.30 µmol, 1 equiv.) in THF (4 mL), then stirred at r.t. for 2 h. After completion of the reaction checked by TLC and LC-MS, the reaction mixture was filtered and concentrated *in vacuo*. The residue was purified by silica-gel flash column chromatography (5% EtOAc in hexane gradient to 30%) to afford **S5** with inseparable byproduct (238.7 mg, 427.97 µmol, 2-step crude yield: 96%).

To a solution of **S5** (238.7 mg, 427.97 µmol, 1 equiv.) in THF (3 mL) in plastic falcon tube at r.t. was added HF/pyridine (330.00 mg, 11.54 mmol, 0.3 mL, 70% purity) in THF (2.7 mL), then stirred at r.t. for

8 h. After completion of the reaction checked by TLC and LC-MS, the reaction mixture was quenched with excess ethoxytrimethylsilane (2.5 mL) and concentrated *in vacuo*. The residue was purified by silica-gel flash column chromatography (10% EtOAc in hexane gradient to 40%) to obtain the desired compound with inseparable byproduct. The crude product was purified by reverse-phase preparative HPLC (20% acetonitrile (ACN) in H_2O with 0.1% trifluoroacetic acid (TFA) gradient to 100%, flow rate: 8 mL/min) to afford **ICM-alkyne** (57.4 mg, 142.99 µmol, 3-step yield: 32%) as a white solid.

^{1}H NMR (500 MHz, DMSO-d_6) δ 9.47 (br s, 1H), 7.69–7.61 (m, 4H), 6.85 (dd, *J* = 8.4, 1.1 Hz, 1H), 6.37 (dd, *J* = 8.4, 2.4 Hz, 1H), 6.24 (d, *J* = 2.4 Hz, 1H), 5.94 (dt, *J* = 4.7, 2.5 Hz, 1H), 5.60–5.59 (m, 1H), 4.32 (s, 1H), 4.24 – 4.10 (m, 2H), 1.53 (s, 3H), 1.50 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 158.19, 153.81, 153.44, 150.61, 135.97, 132.29, 131.89, 126.20, 124.43, 121.27, 114.56, 113.62, 108.56, 104.12, 82.78, 81.77, 79.00, 49.24, 44.00, 27.34, 26.76; HRMS (ESI) *m/z* calcd for $C_{23}H_{20}N_3O_4$ $[M+H]^+$ 402.1448, found: 402.1445.

B. ICMΔOH-alkyne



Scheme 2. Synthesis of ICMΔOH-alkyne. Reagents and conditions: a) $MePPh_3Br$, *t*-BuOK, THF, -40 °C to r.t., 30 min; b) $PhI(OAc)_2$, THF, r.t., 20 min; c) THF, r.t., 2 h.

2-(4-ethynylphenyl)-7,7-dimethyl-5,12b-dihydro-1*H*,7*H*-chromeno[4,3-*c*][1,2,4]triazolo[1,2-*a*]pyridazine-1,3(2*H*)-dione (ICMΔOH-alkyne).

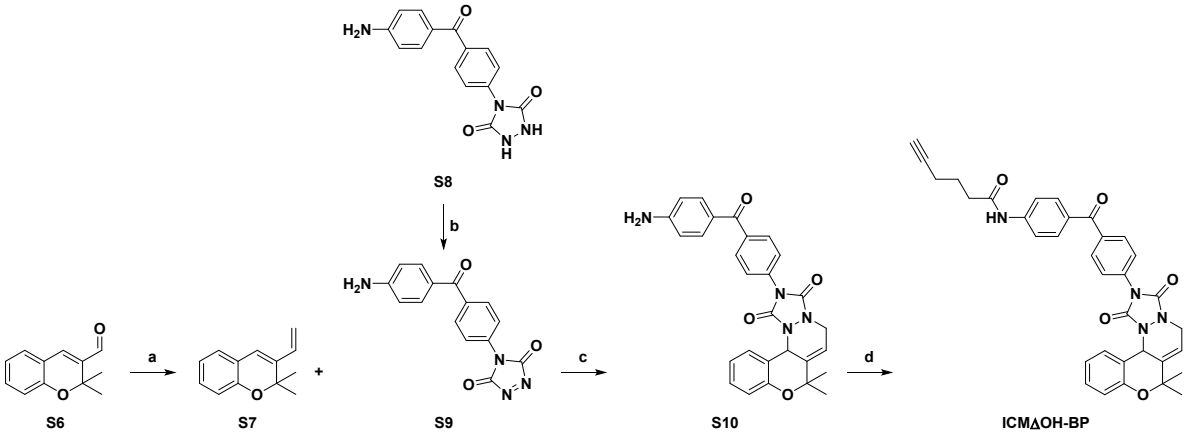
To a mixture of $MePPh_3Br$ (330.60 mg, 925.46 µmol, 2.4 equiv.) and potassium *tert*-butoxide (*t*-BuOK) (103.85 mg, 925.46 µmol, 2.4 equiv.) at -40 °C was added dry THF (2 mL) under Ar(g), then stirred at -40 °C for 30 min. To the reaction mixture at -40 °C was added **S6**[3)] (72.58 mg, 385.61 µmol, 1 equiv.) in dry THF (2 mL), then stirred at r.t. for 30 min. After completion of the reaction checked by TLC and LC-MS, the reaction mixture was quenched with H_2O. The resultant was extracted with EtOAc three times under brine. The combined organic layer was dried over anhydrous Na_2SO_4(s) and concentrated *in vacuo* after filtration. This was used in the next step without any further purification.

To a mixture of **S3** (116.37 mg, 578.42 µmol, 1 equiv.) and $PhI(OAc)_2$ (195.62 mg, 607.34 µmol, 1.05 equiv.) was added THF (3 mL), then stirred at r.t. for 20 min. This was used in the next step without any further purification.

To the reaction mixture of **S4** (115.20 mg, 578.42 µmol, 1.5 equiv.) was added **S7** (71.82 mg, 385.61 µmol, 1 equiv.) in THF (3 mL), then stirred at r.t. for 2 h. After completion of the reaction checked by TLC and LC-MS, the reaction mixture was filtered and concentrated *in vacuo*. The residue was purified by silica-gel flash column chromatography (10% EtOAc in hexane gradient to 30%) to obtain the desired compound. The crude product was purified by reverse-phase preparative HPLC (20% ACN in H_2O with 0.1% TFA gradient to 100%, flow rate: 8 mL/min) to afford **ICMΔOH-alkyne** (74.7 mg, 193.82 µmol, 2-step yield: 50%) as a white solid.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.69–7.67 (m, 2H), 7.65–7.61 (m, 2H), 7.26–7.19 (m, 1H), 7.03 (dt, *J* = 7.6, 1.5 Hz, 1H), 6.96 (td, *J* = 7.5, 1.1 Hz, 1H), 6.90 (dd, *J* = 8.1, 1.1 Hz, 1H), 5.80 (dt, *J* = 4.7, 2.2 Hz, 1H), 5.74–5.70 (m, 1H), 4.29 (ddd, *J* = 16.3, 4.9, 1.3 Hz, 1H), 4.11 (dt, *J* = 16.4, 2.6 Hz, 1H), 3.14 (s, 1H), 1.62 (s, 3H), 1.57 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 153.22, 153.18, 151.00, 138.02, 132.92, 131.60, 129.26, 124.86, 123.99, 123.22, 121.97, 121.73, 118.04, 112.62, 82.73, 79.20, 78.26, 50.55, 44.06, 28.00, 27.01; HRMS (ESI) *m/z* calcd for $C_{23}H_{20}N_3O_3$ $[M+H]^+$ 386.1499, found: 386.1494.

C. ICMΔOH-BP



Scheme 3. Synthesis of ICMΔOH-BP. Reagents and conditions: a) $MePPh_3Br$, *t*-BuOK, THF, -40 °C to r.t., 30 min; b) $PhI(OAc)_2$, THF, r.t., 20 min; c) THF, r.t., 2 h; d) 5-hexynoic acid, $(COCl)_2$, pyridine, DCM, 0 °C, 30 min; e) pyridine, DCM, 0 °C, 30 min.

2-(4-(4-aminobenzoyl)phenyl)-7,7-dimethyl-5,12b-dihydro-1*H*,7*H*-chromeno[4,3-*c*][1,2,4]triazolo[1,2-*a*]pyridazine-1,3(2*H*)-dione (S10).

To a mixture of $MePPh_3Br$ (375.33 mg, 1.05 mmol, 2.4 equiv.) and *t*-BuOK (117.90 mg, 1.05 mmol, 2.4 equiv.) at -40 °C was added dry THF (2 mL) under Ar(g), then stirred at –40 °C for 30 min. To the reaction mixture at -40 °C was added **S6** (82.4 mg, 437.78 µmol, 1 equiv.) in dry THF (2 mL), then stirred at r.t. for 30 min. After completion of the reaction checked by TLC and LC-MS, the reaction

mixture was quenched with H_2O. The resultant was extracted with EtOAc three times under brine. The combined organic layer was dried over anhydrous Na_2SO_4(s) and concentrated *in vacuo* after filtration. This was used in the next step without any further purification.

To a mixture of **S8** (108.09 mg, 364.83 µmol, 1 equiv.) and $PhI(OAc)_2$ (123.39 mg, 383.07 µmol, 1.05 equiv.) was added THF (2 mL), then stirred at r.t. for 20 min. This was used in the next step without any further purification.

To the reaction mixture of **S9** (107.36 mg, 364.83 µmol, 1 equiv.) was added **S7** (81.54 mg, 437.80 µmol, 1.2 equiv.) in THF (2 mL), then stirred at r.t. for 2 h. When the reaction was completed checked by TLC and LC-MS, the reaction mixture was filtered and concentrated *in vacuo*. The residue was purified by MPLC (10% EtOAc in hexane gradient to 60%) to afford **S10** (49.42 mg, 102.85 µmol, Yield: 28%) as a yellow solid.

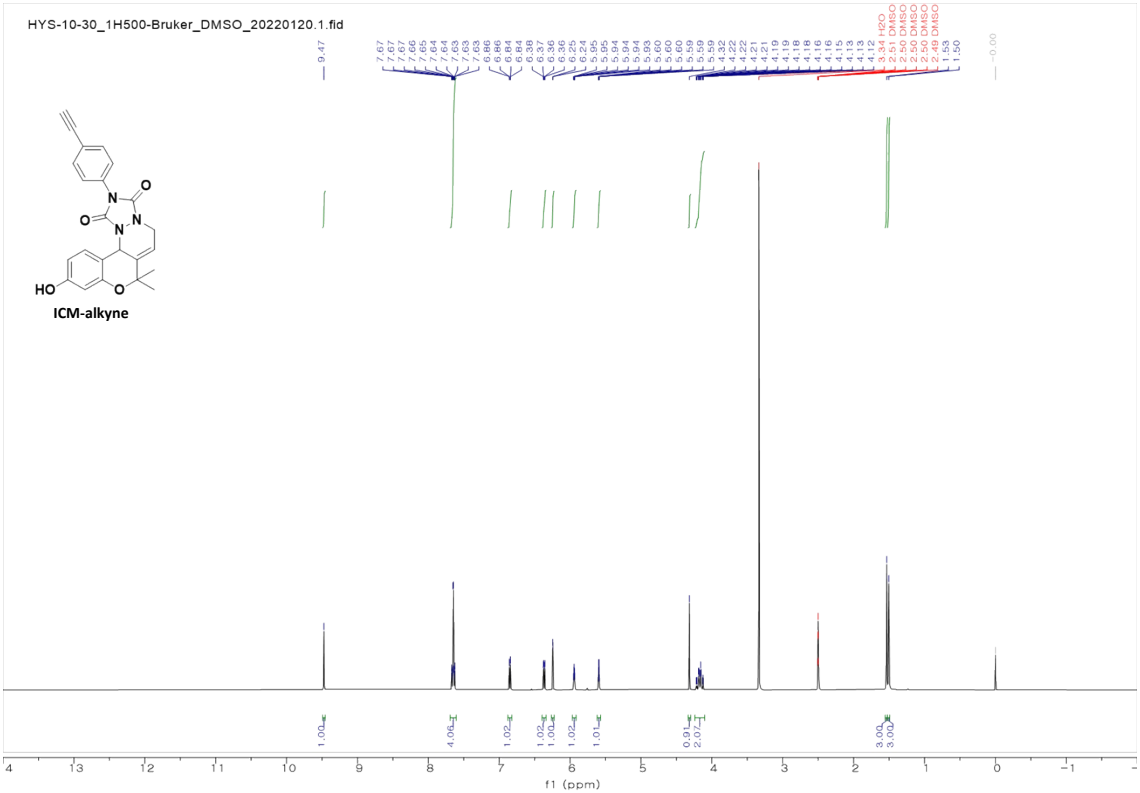
^{1}H NMR (500 MHz, $CDCl_3$) δ 7.90–7.86 (m, 2H), 7.84–7.78 (m, 2H), 7.77–7.71 (m, 2H), 7.25–7.20 (m, 1H), 7.06 (dt, *J* = 7.6, 1.3 Hz, 1H), 6.97 (td, *J* = 7.5, 1.2 Hz, 1H), 6.90 (dd, *J* = 8.1, 1.2 Hz, 1H), 6.71–6.67 (m, 2H), 5.81 (dt, *J* = 4.7, 2.2 Hz, 1H), 5.74 (s, 1H), 4.31 (ddd, *J* = 16.3, 4.9, 1.4 Hz, 1H), 4.19 (br s, 2H), 4.14 (dt, *J* = 16.4, 2.5 Hz, 1H), 1.63 (s, 3H), 1.58 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 193.97, 153.22, 153.17, 151.11, 151.01, 138.26, 137.99, 133.92, 132.96, 130.42, 129.28, 127.06, 124.47, 123.98, 123.21, 121.75, 118.06, 113.67, 112.62, 79.22, 50.57, 44.07, 28.00, 27.02; LRMS (ESI) *m/z* calcd for $C_{28}H_{25}N_4O_4$ $[M+H]^+$ 481.2, found: 481.2.

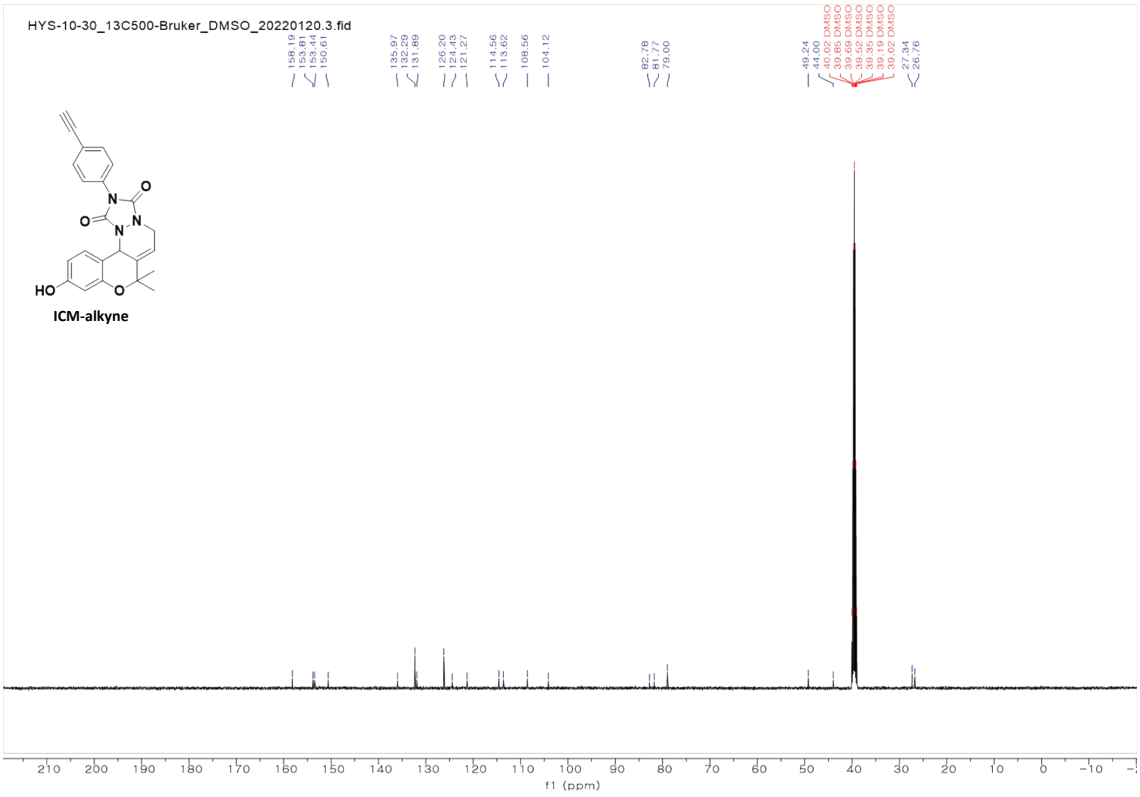
_N_-(4-(4-(7,7-dimethyl-1,3-dioxo-5,12b-dihydro-1_H_,7_H_-chromeno[4,3-_c_][1,2,4]triazolo[1,2-_a_]pyridazin-2(3_H_)-yl)benzoyl)phenyl)hex-5-ynamide (ICMΔOH-BP).

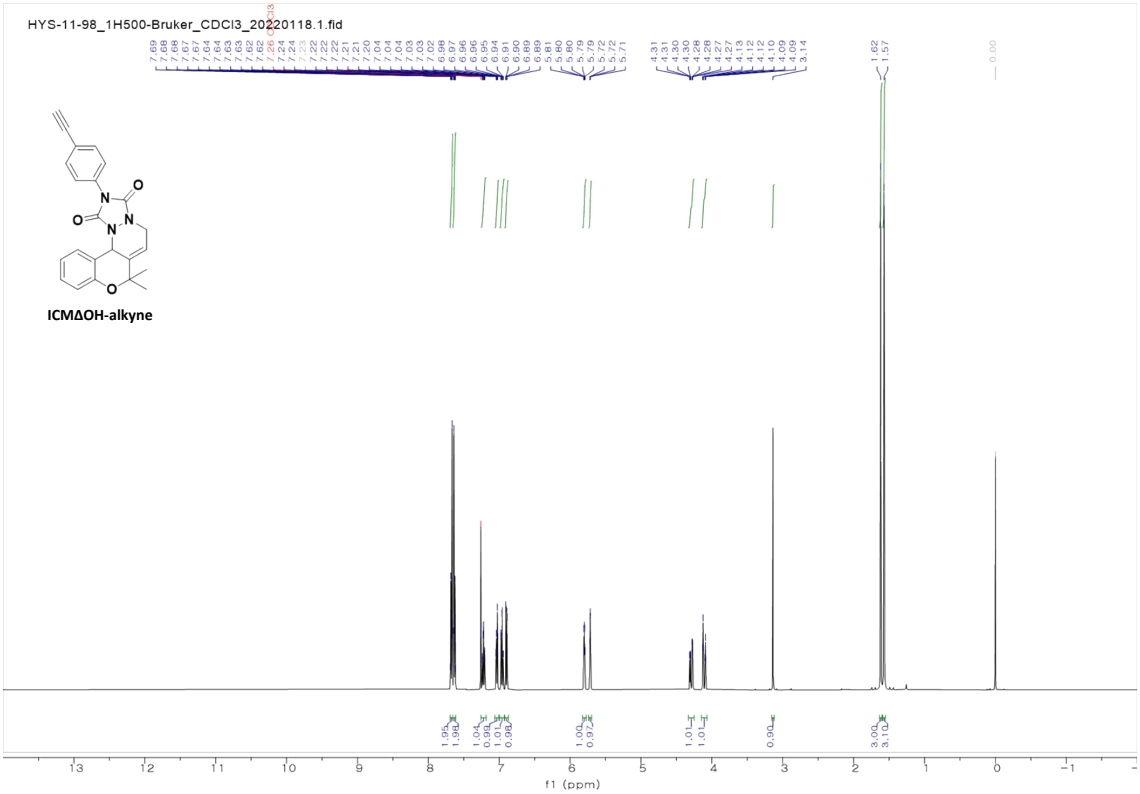
To a solution of 5-hexynoic acid (55.30 mg, 493.22 umol, 53.69 µL, 6 equiv.) in dry DCM (4 mL) at 0 °C was added $(COCl)_2$ (62.60 mg, 493.22 µmol, 42.30 µL, 6 equiv.) slowly under Ar(g), then stirred at 0 °C for 30 min. To the reaction mixture at 0 °C was added **S10** (39.5 mg, 82.20 umol, 1 equiv.) in dry DCM (1 mL) and dry pyridine (78.03 mg, 986.44 µmol, 79.47 µL, 12 equiv.) under Ar(g), then stirred at 0 °C for 30 min. After completion of the reaction checked by TLC and LC-MS, the reaction mixture was concentrated *in vacuo*. The resultant was extracted with DCM three times under brine. The combined organic layer was dried over anhydrous Na_2SO_4(s) and concentrated *in vacuo* after filtration. The residue was purified by MPLC (10% EtOAc in hexane gradient to 50%) to obtain the desired compound. The crude product was purified by reverse-phase preparative HPLC (20% ACN in H_2O with 0.1% TFA gradient to 100%, flow rate: 8 mL/min) to afford **ICMΔOH-BP** (27.5 mg, 47.86 µmol, Yield: 58%) as a white solid.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.92 (d, *J* = 8.4 Hz, 2H), 7.88–7.81 (m, 4H), 7.66 (d, *J* = 8.3 Hz, 2H), 7.59 (br s, 1H), 7.23 (td, *J* = 7.7, 1.7 Hz, 1H), 7.05 (d, *J* = 7.6 Hz, 1H), 6.97 (t, *J* = 7.5 Hz, 1H), 6.90 (d, *J* = 8.1 Hz, 1H), 5.81 (dt, *J* = 4.8, 2.2 Hz, 1H), 5.74 (s, 1H), 4.36–4.27 (m, 1H), 4.14 (dt, *J* = 16.4, 2.6 Hz, 1H), 2.56 (t, *J* = 7.3 Hz, 2H), 2.34 (td, *J* = 6.7, 2.6 Hz, 2H), 2.02 (t, *J* = 2.7 Hz, 1H), 1.97 (p, *J* = 7.0 Hz, 2H), 1.63 (s, 3H), 1.58 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 194.32, 170.78, 153.23, 153.03, 150.91, 142.00, 138.02, 137.11, 134.69, 132.62, 131.64, 130.76, 129.31, 124.55, 123.94, 123.18, 121.77, 118.78, 118.09, 112.56, 83.28, 79.23, 69.58, 50.61, 44.06, 35.99, 28.02, 27.03, 23.68, 17.74; HRMS (ESI) *m/z* calcd for $C_{34}H_{31}N_4O_5$ $[M+H]^+$ 575.2289, found: 575.2281.

^{1}H and ^{13}C NMR Spectra



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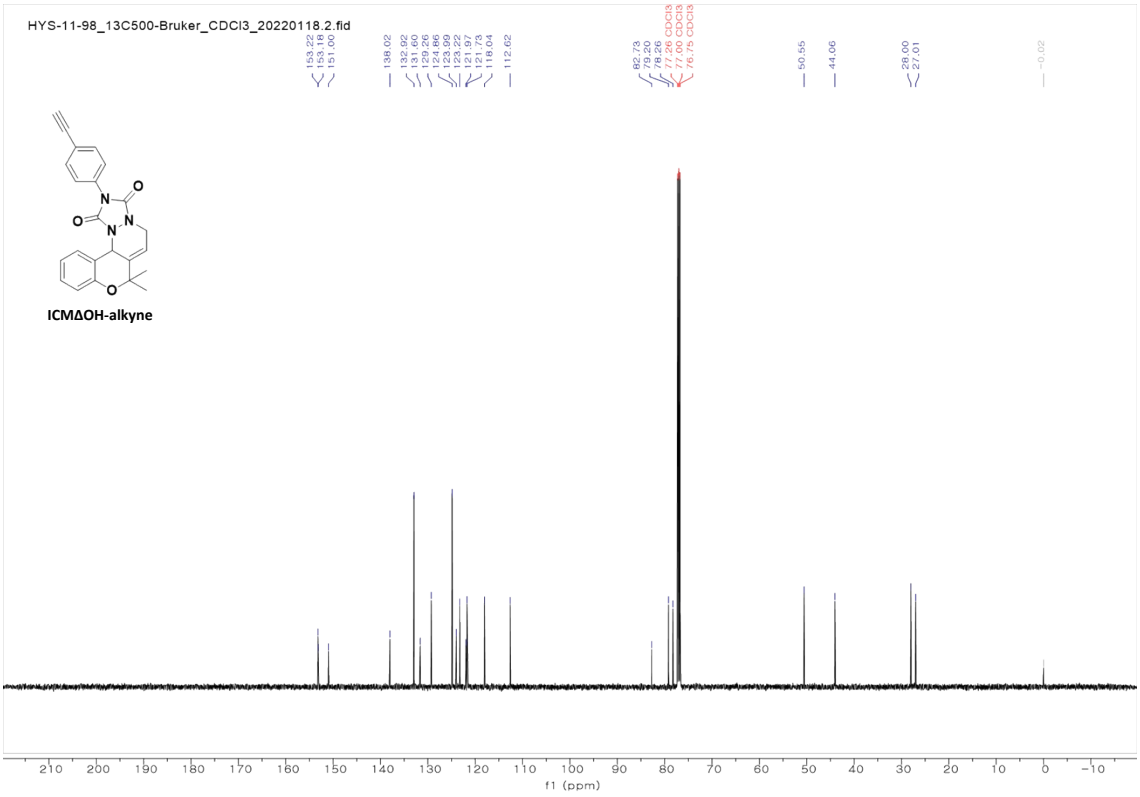
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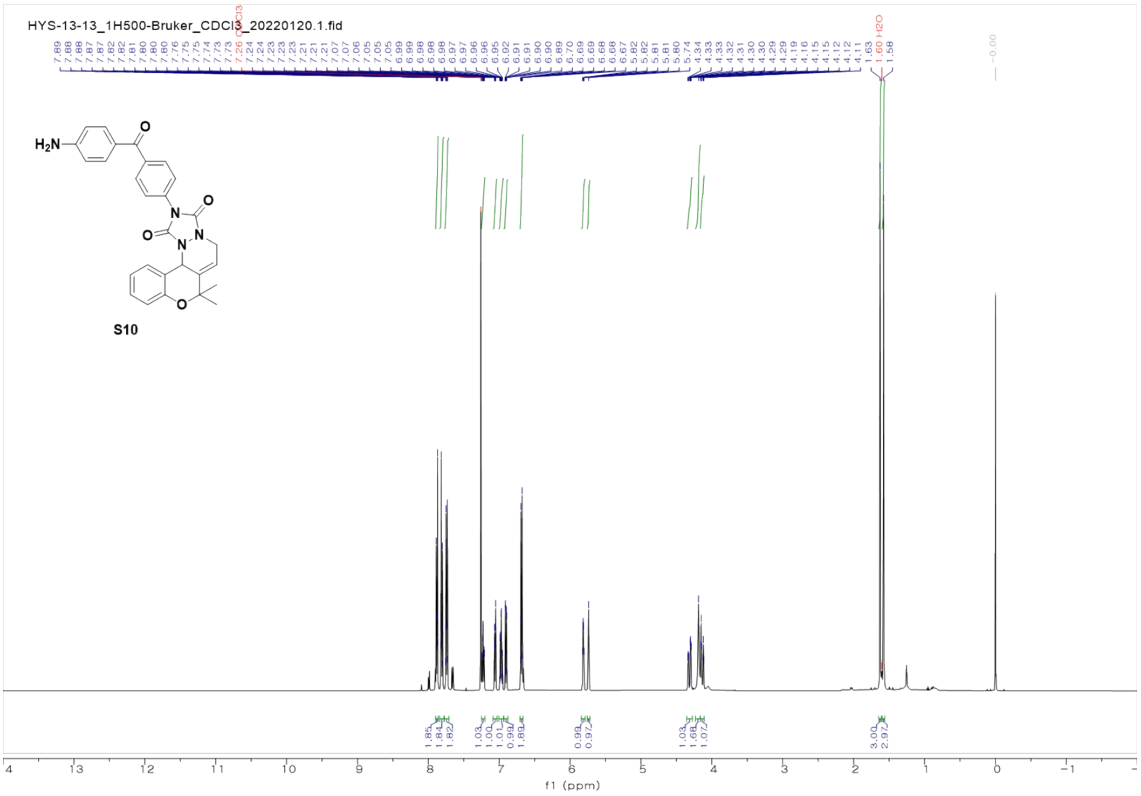
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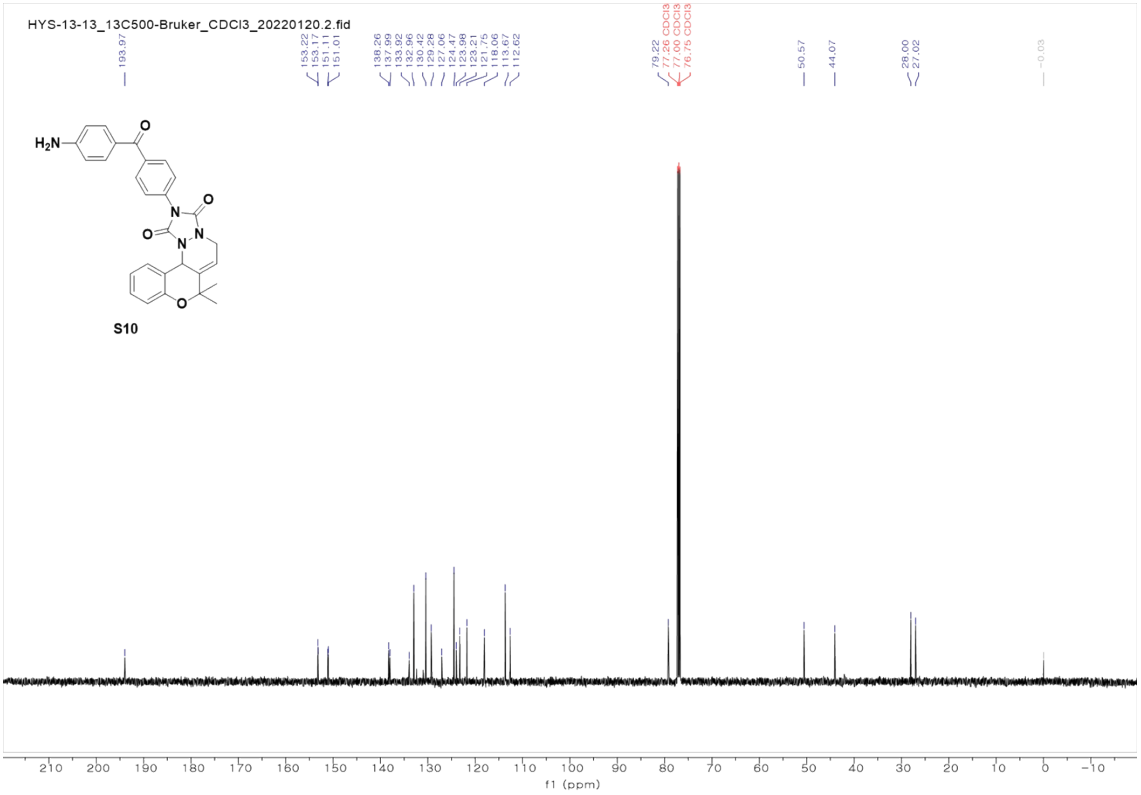
S10
f1 (ppm)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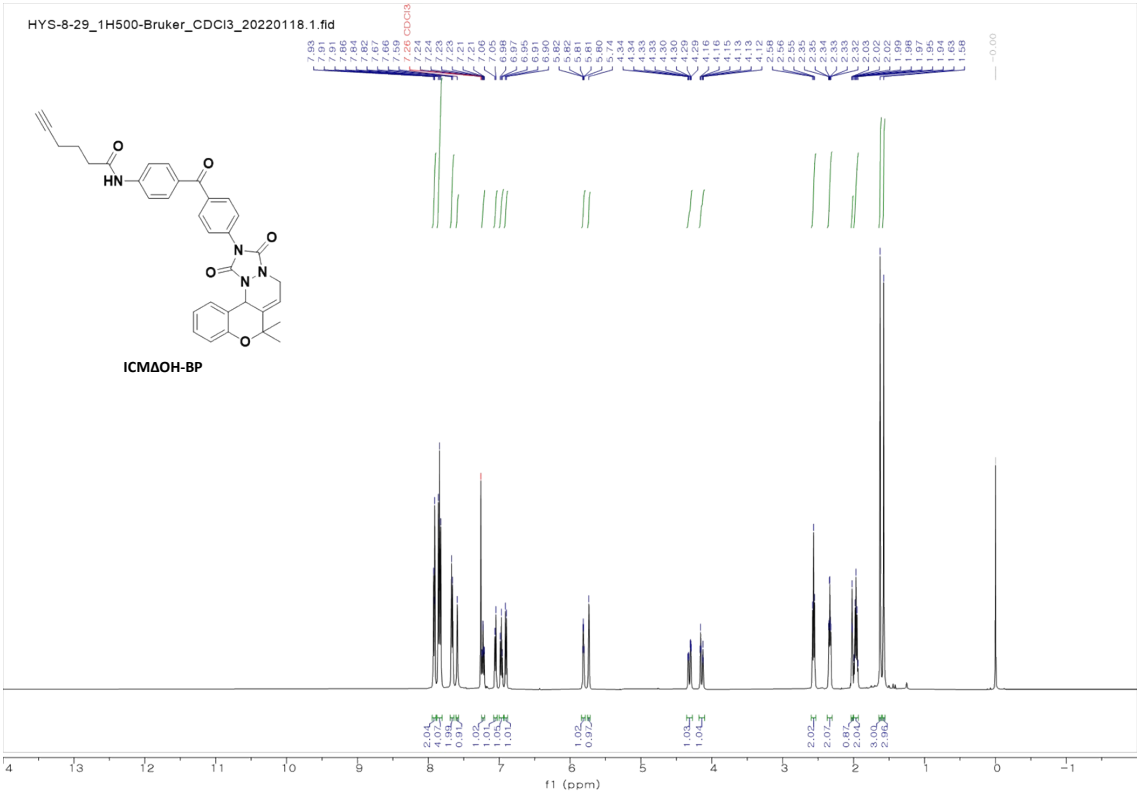
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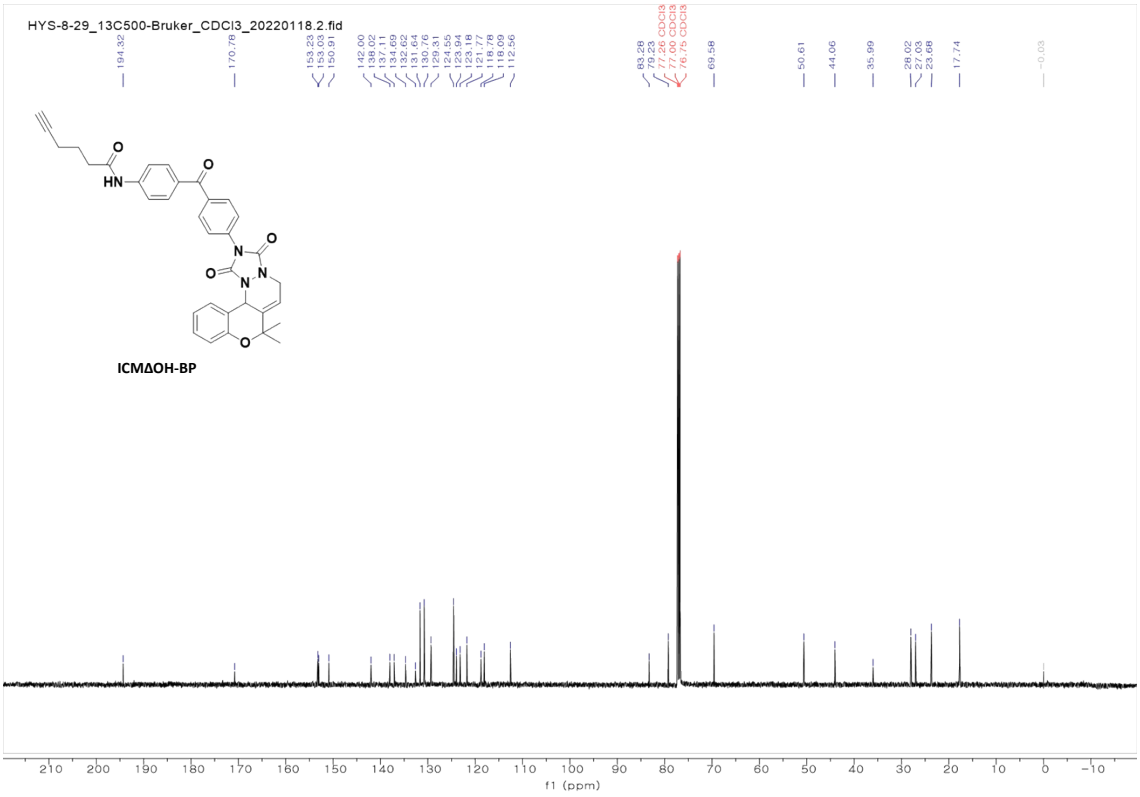
f1 (ppm)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f1 (ppm)

References

1. J. Park, S. Oh and S. B. Park, *Angew. Chem. Int. Ed.*, 2012, **51**, 5447–5451.

2. J.-E. Choi, J.-J. Lee, W. Kang, H. J. Kim, J.-H. Cho, P.-L. Han and K.-J. Lee, *Mol. Cell. Proteomics*, 2018, **17**, 1803–1823.