Electronic Supplementary Material (ESI) for ChemComm.

Target profiling of zerumbone using a novel cell-permeable clickable probe and quantitative chemical proteomics

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Note: Supporting Table 1 has been provided as a separate Excel file (ESI† Supporting Table 1)

1. Supporting Figures and Tables

gene symbol	significant enrichment at 75/100/150 µM zerumbone treatment	localisation	major protein functions
DFNA5	+ + +	cytoplasm	apoptosis, cancer and cell survival.
CDA	+ + +	cytoplasm, nucleus	scavenges cytidine and 2'-deoxycytidine for UMP synthesis
UVRAG	+ + +	endosome, lysosome	DNA repair, positive regulation of autophagy, regulation of intrinsic pathway of apoptosis
LCMT1	+ + +	cytoplasm	c-terminal protein methylation, regulation of apoptosis
NT5DC1	+ + +	cytosol	hydrolase
CPPED1	+ + +	cytoplasm	protein phosphatase of Akt-family kinases, blocks cell cycle progression and promotes apoptosis
NT5CD2	+ + +	mitochondrion	hydrolase
FAM114A1	+ + +	cytoplasm	neuronal cell development
GCLC	+ + +	cytoplasm, cytosol	glutathione biosynthesis, cell redox homeostasis, apoptotic mitochondrial changes
MLKL	+ + +	cell membrane, cytoplasm	TNF-induced necroptosis
NR3C1	+ + +	cytoplasm, mitochondrion, nucleus	transcription regulation, affects inflammatory responses and cellular proliferation
ATXN10	+ + +	cytoplasm	necessary for the survival of cerebellar neurons, apoptosis
TMPO	+ + +	nucleus	regulation of transcription
RPS6KA1	+ + +	nucleus, cytoplasm	serine/threonine kinase that regulate many cellular processes including growth, motility, survival and proliferation.

BRAT1	+ + +	nucleus	cellular response to DNA damage
MCMBP	+ + +	nucleus	cell cycle, cell division, DNA replication, mitosis
MAGED2	+ + +	cytosol, nucleus	tumor antigen, protects melanoma cells from apoptosis induced by TRAIL
UQCRC1	+ + +	mitochondrion	electron transport
SYNCRIP	+ + +	cytoplasm, ER, microsome, nucleus, spliceosome	translation regulation, mRNA processing and splicing, host-virus interaction
DUT	+ + +	mitochondrion, nucleus	nucleotide metabolism
HELLS	+ +	nucleus	thought to be involved in cellular proliferation and leukemogenesis
AIP	+ +	cytoplasm	regulate expression of many xenobiotic metabolizing enzymes
UQCRC1	+ +	mitochondrion, cytosol	aerobic respiration
PSME2	+ +	cytosol, nucleus	immunoproteasome assembly
HNRNPF	+ +	nucleus, spliceosome	mRNA processing and mRNA splicing
CKAP5	+ +	cytoplasm, cytoskeleton	regulates microtubule dynamics, involved in cell cycle, cell division and mitosis
EIF3F	+ +	cytoplasm	protein biosynthesis
IPO4	+	cytosol, nucleus	nuclear protein import
MOCOS	+	cytosol	sulfuration of molybdenum cofactor
ARFIP2	+	cytoplasm	putative target protein of ADP-ribosylation factor

TK1	+	cytosol	nucleoside kinase
PDE3A	+	cytosol	cGMP-mediated signaling, negative regulation of apoptosis
PLEKHA2	+	plasma membrane	positive regulation of cell-matrix adhesion
DDB2	+	nucleus	repair of UV light-damaged DNA
HNRNPR	+	nucleus	precursor mRNA processing in the nucleus
HSPB1	+	cytoskeleton, nucleus, cytosol, plasma membrane	stress resistance, actin organization, negative regulation of apoptosis, regulation of I-kappaB kinase/NF-kappaB signaling
CKAP5	+	cytoskeleton, cytosol, nucleus, plasma membrane	microtubule dynamics and organization
HSPB8	+	cytoplasm, nucleus	temperature-dependent chaperone activity
SPAG5	+	cytoskeleton, nucleus, cytosol, mitochondrion	functional and dynamic regulation of mitotic spindled
NUBP2	+	cytoplasm	maturation and assembly of extramitochondrial Fe-S proteins
TIGAR	+	cytosol	glycolysis inhibitor, may protect cells against reactive oxygen species and apoptosis induced by tp53
PCBP1	+	nucleus, cytoskeleton, cytosol	single-strand nucleic acid binding, gene expression and RNA splicing
MYBBP1A	+	nucleus	transcription regulation via interaction with DNA-binding proteins

DYNC1LI1	+	cytoplasm	regulates dynein function
RPS6KA3	+	cytosol, nucleus	serine/threonine kinase that regulate many cellular processes including growth, motility, survival and proliferation.
GOPC	+	golgi apparatus, plasma membrane, cytosol	intracellular protein trafficking and degradation, may play a role in autophagy
RARS	+	cytoplasm	protein biosynthesis
IPO5	+	cytoplasm, nucleus	protein transport, host-viral interaction
FBXO30	+	cytosol, nucleus	substrate-recognition of SCF-type E3 ubiquitin ligase complex
TBC1D15	+	extracellular region	regulation of intracellular trafficking, GTPase activator for RAB7A
AARS	+	cytoplasm	catalysis of attachment of alanine to tRNA(Ala)
TBC1D4	+	cytoplasm, extracellular vesicular exosome	may act as GTPase activator for RAB2A, RAB8A, RAB10 and RAB14.
USP48	+	nucleus, mitochondrion, cytoplasm	hydrolysis of peptide bond at the C-terminal glycine of ubiquitin, involved in the processing of poly-ubiquitin precursors and ubiquitinated proteins, possibly involved in the regulation of NF-kappa-B activation by TNF receptor superfamily.
ANXA2	+	extracellular space, plasma membrane	calcium ion binding, possibly involved in heat-stress response
DHX15	+	nucleolus	pre-mRNA processing
TMOD3	+	cytoplasm, cytoskeleton	formation of short actin protofilament

AMMECR1	+	nucleus, plasma membrane, cytosol	unknown
RRP1B	+	cytosol, nucleus	ribosomal RNA processing.
IMPDH1	+	nucleus, cytosol	regulation of cell growth, may play a role in the development of malignancy and growth of some tumors.
RRM1	+	cytosol, nucleus	provides precursors for DNA synthesis
MMS19	+	cytoskeleton, cytosol, nucleus	mediates incorporation of iron-sulfur cluster into apoproteins specifically involved in DNA metabolism and genome integrity
CBS	+	cytosol, nucleus	regulation of hydrogen sulfide.

Supporting Table 2: Identified top targets of zerumbone in HeLa cells. Proteins that showed statistically significant [Threshold P value < 0.05, n = 3 (three biological replicates)] and more than one fold in log2 scale enrichment in the H/L ratio at all three tested concentrations of zerumbone (75, 100 and 150 µM) compared to the no-zerumbone treatment samples are indicated with triple plus (+ + +) signs, while proteins with (+ +) and (+) signs correspond to additional targets significantly enriched at 100 and 150 µM concentrations of zerumbone treatment respectively.

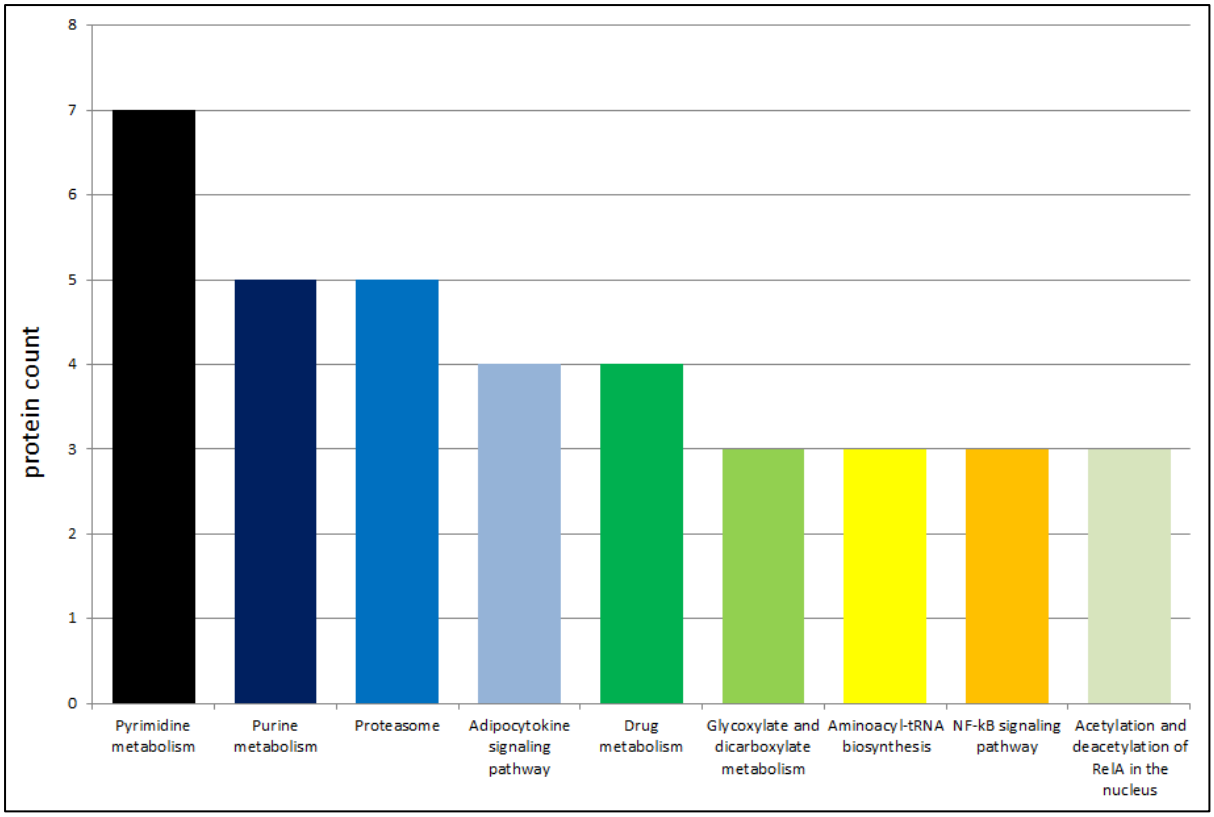


Fig. S1: Key biological pathways where zerumbone targets are involved revealed by DAVID bioinformatics analysis[1,2] of 151 proteins that showed concentration-dependent competition from zerumbone in the Spike-in-SILAC-Yn-Zer labelling experiment.

Biological pathway	Protein count	P value	Proteins targets identified
Pyrimidine metabolism	7	0.00067	1) CTP synthase 1 2) Cytidine deaminase 3) Thymidine kinase, cytosolic 4) CAD protein 5) Deoxyuridine 5'-triphosphate nucleotidohydrolase, mitochondrial 6) Nucleoside diphosphate kinase A 7) Ribonucleoside-diphosphate reductase large subunit
Proteasome	5	0.0019	1) 26S proteasome non-ATPase regulatory subunit 13 2) 26S proteasome non-ATPase regulatory subunit 14 3) Proteasome activator complex subunit 1 4) Proteasome activator complex subunit 2 5) Proteasome activator complex subunit 3
Glycoxylate and dicarboxylate metabolism	3	0.012	1) Citrate synthase, mitochondrial 2) Aconitate hydratase, mitochondrial 3) Monofunctional C1-tetrahydrofolate synthase, mitochondrial
Drug metabolism	4	0.013	1) Cytidine deaminase 2) Thymidine kinase, cytosolic 3) Inosine-5'-monophosphate dehydrogenase 1

			4) Inosine-5'-monophosphate dehydrogenase 2
Acetylation and deacetylation of RelA in the nucleus	3	0.02	1) Transcription factor p65 (NF-kappa-B) 2) FAS-associated death domain protein 3) Inhibitor of nuclear factor kappa-B kinase subunit alpha
Adipocytokine signaling pathway	4	0.04	1) Transcription factor p65 (NF-kappa-B) 2) Signal transducer and activator of transcription 3 3) 5'-AMP-activated protein kinase catalytic subunit alpha-1 4) Inhibitor of nuclear factor kappa-B kinase subunit alpha
Epithelial cell signaling in Helicobacter pylori infection	4	0.042	1) Transcription factor p65 (NF-kappa-B) 2) Inhibitor of nuclear factor kappa-B kinase subunit alpha 3) 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-1 4) V-type proton ATPase catalytic subunit A
NF-kB signaling pathway	3	0.054	1) Transcription factor p65 (NF-kappa-B) 2) FAS-associated death domain protein 3) Inhibitor of nuclear factor kappa-B kinase subunit alpha
Aminoacyl-tRNA biosynthesis	3	0.079	1) Alanyl-tRNA synthetase 2) Arginyl-tRNA synthetase 3) Glutaminyl-tRNA synthetase
Purine metabolism	5	0.096	1) Inosine-5'-monophosphate dehydrogenase 1 2) Inosine-5'-monophosphate dehydrogenase 2 3) Nucleoside diphosphate kinase A 4) cGMP-inhibited 3',5'-cyclic phosphodiesterase A 5) Ribonucleoside-diphosphate reductase large subunit

Supporting Table 3: Biological pathways with number of protein targets matched and its p-value identified in DAVID bioinformatics analysis ($p < 0.1$). The identities of targets are also indicated.

Biological processes	**Protein count**	**P value**	**Proteins targets identified**
Nitrogen compound biosynthetic processes	12	0.00023	1) Cytidine deaminase 2) CTP synthase 1 3) V-type proton ATPase catalytic subunit A 4) Inosine-5'-monophosphate dehydrogenase 1 5) Inosine-5'-monophosphate dehydrogenase 2 6) CAD protein 7) Cystathionine beta-synthase 8) Molybdenum cofactor sulfurase 9) Protoporphyrinogen oxidase 10) Ribonucleoside-diphosphate reductase large subunit 11) DNA mismatch repair protein Msh2 12) Nucleoside diphosphate kinase A
Negative regulation of apoptosis	10	0.0058	1) BCL2-associated athanogene 3 2) Alanyl-tRNA synthetase 3) Glutamate-cysteine ligase, catalytic subunit

			4) Glutamate-cysteine ligase, modifier subunit 5) Heat shock protein beta-1 6) Lymphoid-specific helicase 7) DNA mismatch repair protein Msh2 8) Nucleoside diphosphate kinase A 9) cGMP-inhibited 3',5'-cyclic phosphodiesterase A 10) Transcription factor p65 (NF-kappa-B)
Cell cycle process	13	0.0064	1) Cytoskeleton-associated protein 5 2) Dynactin subunit 2 3) Lymphoid-specific helicase 4) Interleukin enhancer-binding factor 3 5) DNA mismatch repair protein Msh2 6) 26S proteasome non-ATPase regulatory subunit 13 7) 26S proteasome non-ATPase regulatory subunit 14 8) Proteasome activator complex subunit 1 9) Proteasome activator complex subunit 2 10) Proteasome activator complex subunit 3 11) Sperm-associated antigen 5 12) Structural maintenance of chromosomes protein 2 13) Thyroid receptor-interacting protein 13
Cytoskeleton organization	11	0.0076	1) Arfaptin-2 2) LIM and calponin homology domains-containing protein 1 3) Rho-associated protein kinase 2 4) Cytoskeleton-associated protein 5 5) Dynactin subunit 2 6) Dynein light chain 1, cytoplasmic 7) Microtubule-associated protein 1B 8) Microtubule-associated protein 7 9) Palladin 10) Sperm-associated antigen 5 11) Vasodilator-stimulated phosphoprotein
Mitotic cell cycle	10	0.0077	1) Cytoskeleton-associated protein 5 2) Dynactin subunit 2 3) Lymphoid-specific helicase 4) 26S proteasome non-ATPase regulatory subunit 13 5) 26S proteasome non-ATPase regulatory subunit 13 6) Proteasome activator complex subunit 1 7) Proteasome activator complex subunit 2 8) Proteasome activator complex subunit 3 9) Sperm-associated antigen 5 10) Structural maintenance of chromosomes protein 2
Regulation of programmed cell death	16	0.0085	1) BCL2-associated athanogene 3 2) FAS-associated death domain protein 3) NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial 4) Alanyl-tRNA synthetase 5) Cystatin-B 6) Dynein light chain 1, cytoplasmic 7) Glutamate--cysteine ligase catalytic subunit 8) Glutamate--cysteine ligase modifier subunit 9) Heat shock protein beta-1 10) Lymphoid-specific helicase 11) DNA mismatch repair protein Msh2

			12) Nucleoside diphosphate kinase A 13) Glucocorticoid receptor 14) cGMP-inhibited 3',5'-cyclic phosphodiesterase A 15) Proteasome activator complex subunit 3 16) Transcription factor p65 (NF-kappa-B)
Cellular protein catabolic processes	13	0.011	1) F-box only protein 30 2) E3 ubiquitin-protein ligase HUWE1 3) DNA damage-binding protein 2 4) 26S proteasome non-ATPase regulatory subunit 13 5) 26S proteasome non-ATPase regulatory subunit 14 6) Proteasome activator complex subunit 1 7) Proteasome activator complex subunit 2 8) Proteasome activator complex subunit 3 9) E3 ubiquitin-protein ligase RNF14 10) Ubiquitin carboxyl-terminal hydrolase 48 11) E2/E3 hybrid ubiquitin-protein ligase UBE2O 12) Ubiquitin-like modifier-activating enzyme 6 13) Transcription factor p65 (NF-kappa-B)
Negative regulation of macromolecule metabolic processes	14	0.019	1) Translation initiation factor eIF-2B subunit gamma 2) Glutamate--cysteine ligase catalytic subunit 3) Lymphoid-specific helicase 4) Interleukin enhancer-binding factor 3 5) DNA mismatch repair protein Msh2 6) COUP transcription factor 1 7) 26S proteasome non-ATPase regulatory subunit 13 8) 26S proteasome non-ATPase regulatory subunit 14 9) Proteasome activator complex subunit 1 10) Proteasome activator complex subunit 2 11) Proteasome activator complex subunit 3 12) Transcriptional activator protein Pur-beta 13) Signal transducer and activator of transcription 3 14) Transcription factor p65 (NF-kappa-B)

Supporting Table 4: Table showing the identity of protein targets and their enrichment P value in the DAVID bioinformatics analysis for biological processes (threshold P value < 0.05). Only selected list of biological processes from the full analysis are presented.

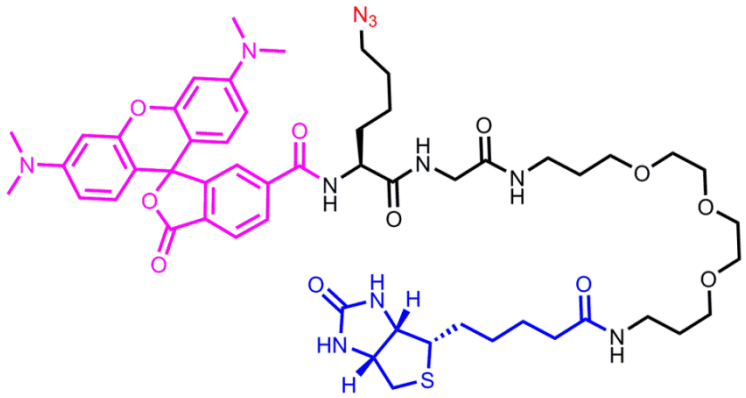
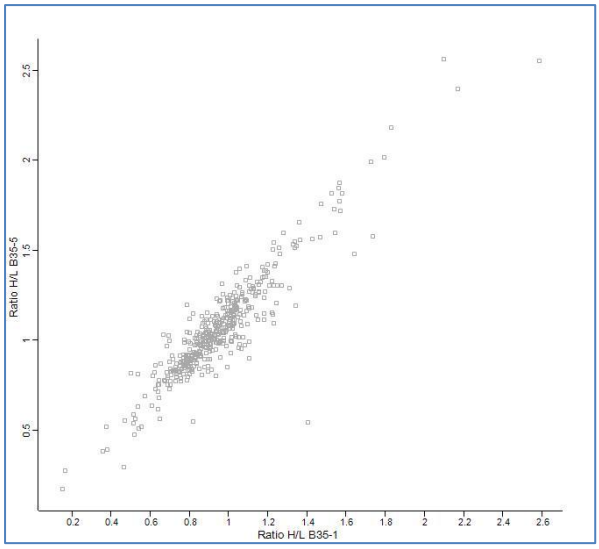
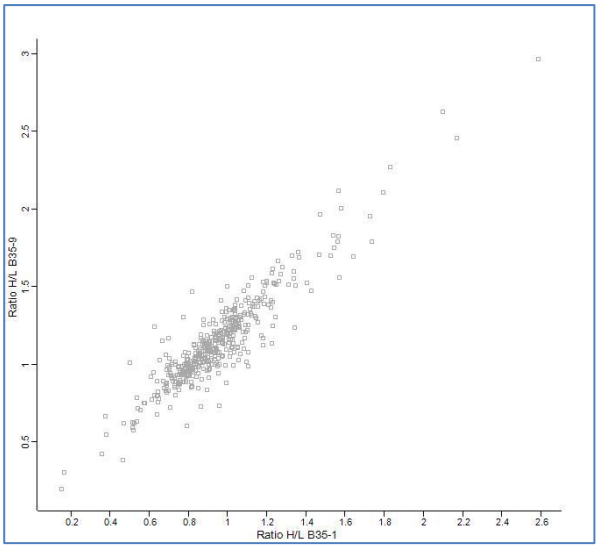


Fig. S2 Chemical structure of the capture reagent (AzTB)


Ratio H/L B35-5
Ratio H/L B35-1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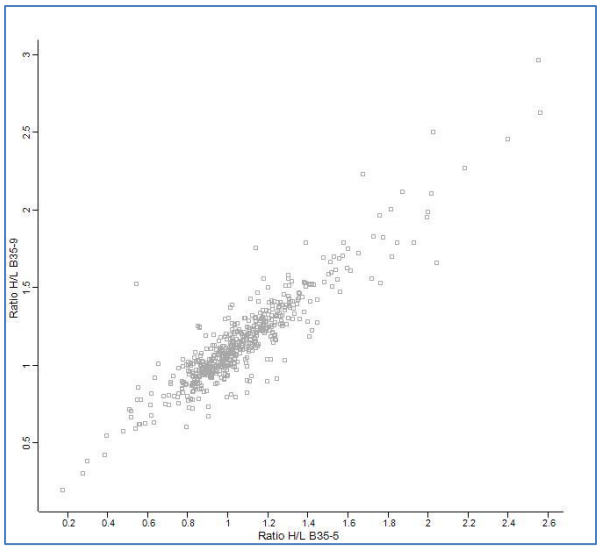
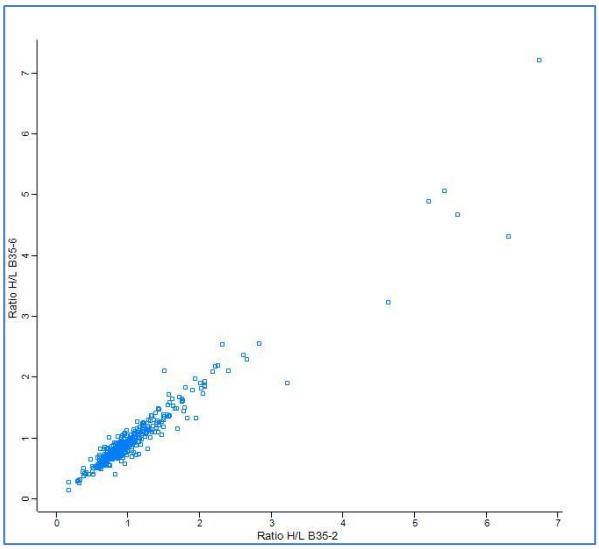
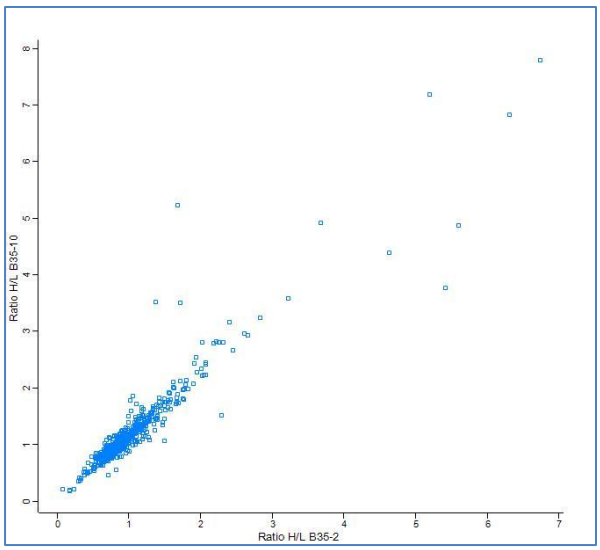
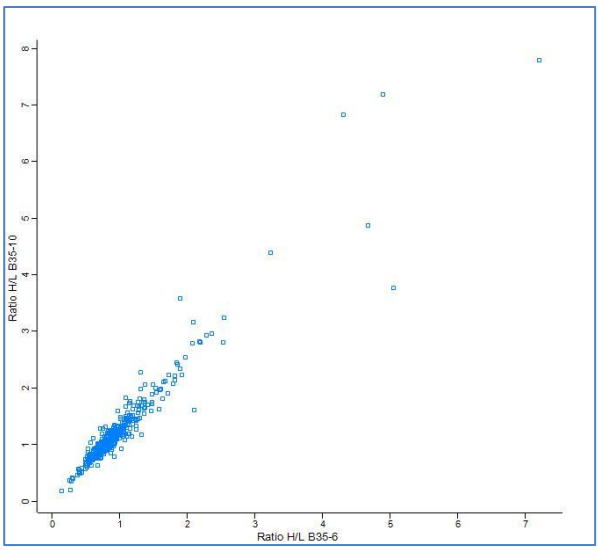
Ratio H/L B35-1


Ratio H/L B35-9
Ratio H/L B35-5


Ratio H/L B35-6
Ratio H/L B35-2


Ratio H/L B35-10
Ratio H/L B35-2


Ratio H/L B35-10
Ratio H/L B35-6

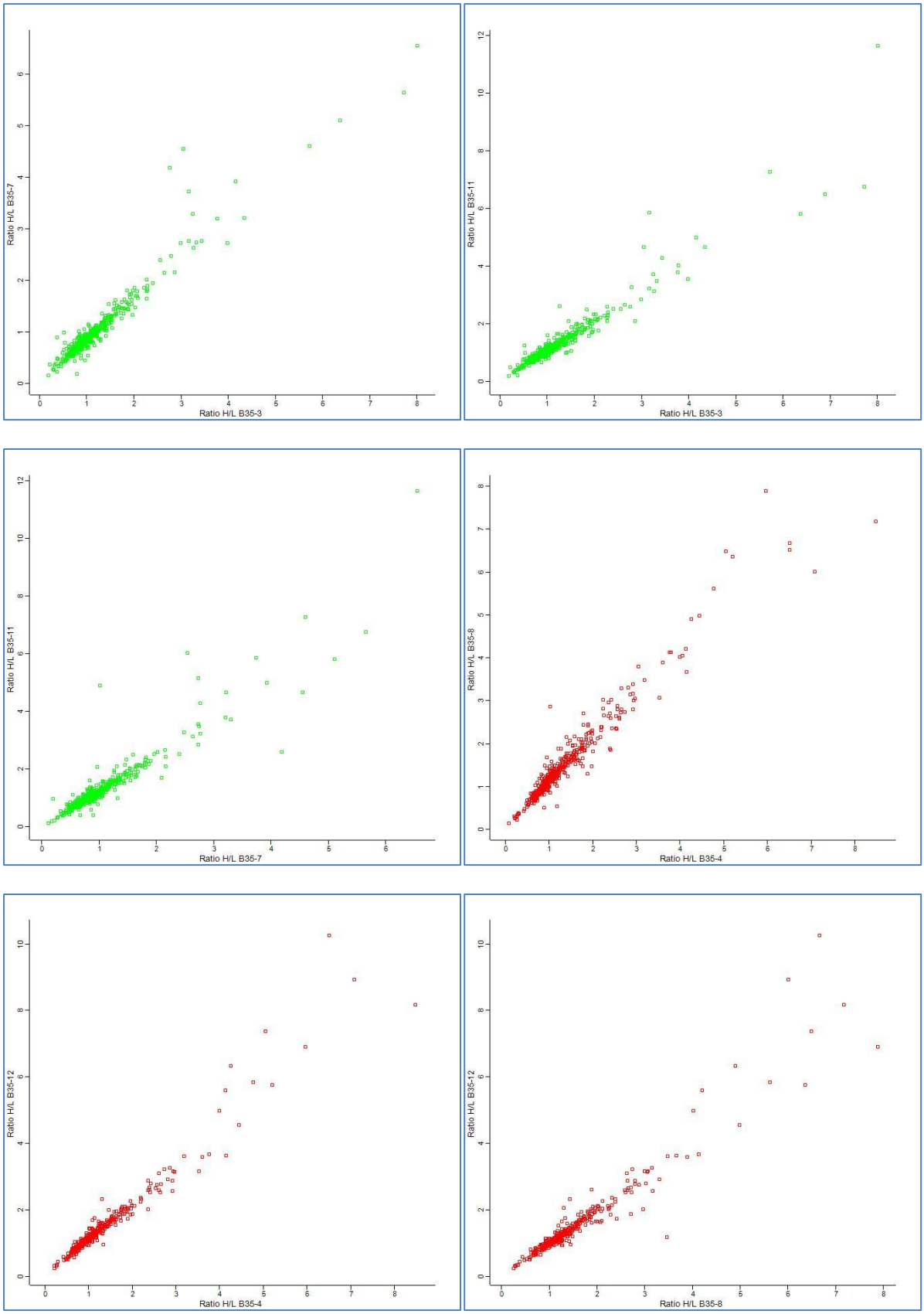


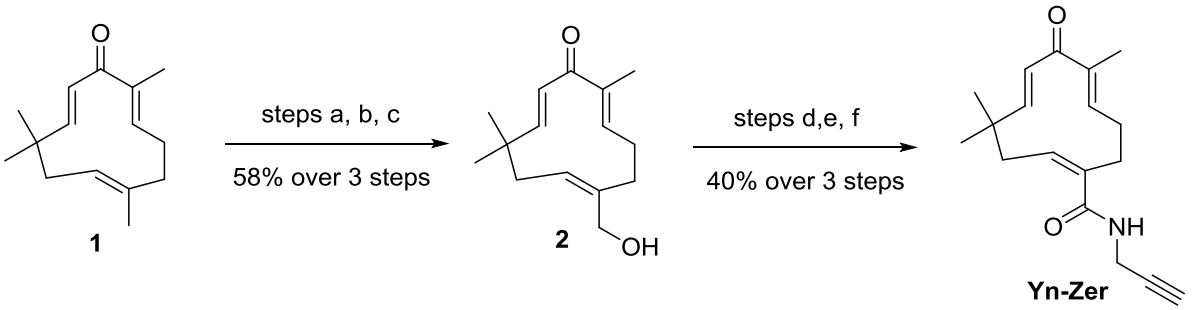
Fig. S3 Scatter plots between H/L ratios across the biological replicates (n = 3) at each tested concentration of zerumbone. (Grey = 0, Blue = 75, Green = 100 and Red = 150 µM respectively of zerumbone treated samples)

2. Supporting methods

2. 1. General information

All chemical were purchase from commercial suppliers and used without further purification. The following abbreviations were used: NBS (N-bromosuccinimide), *t*Bu (tertiary butyl), DMF (dimethylformamide), DMSO (dimethyl sulfoxide), DIEA (diisopropylethyl amine), HATU (1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate**)**, TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl), BAIB ([bis(acetoxy)iodo]benzene), DMEM **(**dulbecco's modified eagle's medium), FBS (fetal bovine serum), TCEP (tris(2-carboxyethyl) phosphine hydrochloride), TBTA (tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine), SDS (sodium dodecyl sulfate), EDTA (ethylenediaminetetraacetic acid), and DTT (dithiothreitol), FA (formic acid), TFA (trifluoroacetic acid), PAGE (polyacrylamide gel electrophoresis), ESI (electrospray ionisation), PBS (phosphate buffered saline), HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) , CuAAC (copper catalyzed azide-alkyne cycloaddition). Ultrapure water was obtained from MilliQ® Millipore water purification system. Thin Layer Chromatography was performed on Merck pre-coated Silica plates (Aluminium oxide 60 F254, Merck). Spots were visualized by UV light (operating at 254 nm), and using appropriate stain. Flash column chromatography was carried out either by using hand-made silica columns with Merck Silica 60Å, or on Isolera (Biotage, UK) automated apparatus with fraction collector equipped with SNAP cartridges columns (Biotage, UK). NMR spectra were recorded on 400MHz Bruker instruments and were referenced to residual solvent signals. Data are presented as follows: chemical shifts δ (ppm); multiplicity s = singlet, d = doublet, t = triplet, m = multiplet, br = broad signal; coupling constants in Hz = Hertz. High resolution mass spectrometry (HRMS) was performed on Waters LCT Premier Spectrometer. Analytical reverse phase-LC-MS was carried out on a Waters 2767 system equipped with a photodiode array detector for the LC and a mass spectrometer with electrospray ionisation source. A flow rate of 1.2 mL/min with a gradient of water and methanol with 0.05% formic acid was used for the analytical LC-MS. For quantitative proteomics (spike-in SILAC) R10K8 and R0K0 DMEM media were purchased from Dundee Cell Products. Cell dissociation buffer (enzyme free, PBS-based), obtained from Gibco (Life technologies) was used instead of trypsin to detach the cells before passaging. Dialyzed FBS was obtained from Sigma-Aldrich. For proteomics, all buffers were filtered using a 0.2μM filter. Low binding tubes (Protein LoBind tubes, Eppendorf) were used to carry out affinity enrichment and all subsequent steps.

2. 2. Synthesis of the clickable probe, Yn-Zer



a) NBS, CH_3CN/H_2O (1:1), r.t, 1min; b) Sodium acetate, DMF, r.t, 16h; c) NaOH, H_2O, r.t, 6h; d) TEMPO/BAIB, CH_3CN/H_2O, r.t, 16h; e) $NaClO_2$, NaH_2PO_4, t-BuOH, H_2O, 2-methyl-2-butene, r.t, 6h; f) Propargylamine, HATU, DIPEA, DMF, r.t, 1h

Supporting Scheme 1: Synthesis of the clickable probe **Yn-Zer**

(2E,6Z,10E)-6-(hydroxymethyl)-2,9,9-trimethylcycloundeca-2,6,10-trien-1-one (**2**)

The intermediate alcohol **2** was synthesized as reported previously.[3] Briefly, to a solution of zerumbone (**1)** (50mg, 0.23mmols) in a 1:1 (v/v) mixture of acetonitrile and water (0.5mL each) was added N-bromosuccinimide (49.2 mg, 0.276mmoles) and the reaction mixture was stirred at room temperature for 1min, upon which a white precipitate was formed. To the reaction mixture was quickly added 10mL water and the turbid solution was filtered. The bromosubstituted product (2E,10E)-7-bromo-2,9,9-trimethyl-6-methylidenecycloundeca-2,10-dien-1-one was obtained as a white solid, which was dried and used immediately for the next step without any purification. Yield = 50mg. LC-MS (ESI) *m/z* (calculated for $C_{15}H_{22}BrO$) = 297.08 $[M+H]^+$ *m/z* (found) = 297.24 $[M+H]^+$, 299.26 $[M+H]^+$, 319.22 $[M+Na]^+$, 321.23 $[M+Na]^+$. The bromosubstituted product (35mg, 0.118mmols) was then dissolved in DMF (1mL). To this was added sodium acetate (9.7mg, 0.118mmols) and the reaction was left to stir at room temperature for 16 hrs. The reaction mixture was then diluted with dichloromethane (20mL) and extracted with water (2 X 10mL). The organic layer was dried over anhydrous sodium sulphate and concentrated. Purification on silica gel flash column chromatography afforded 30mg (0.108mmols) of acetate substituted zerumbone, [*(1Z,5E,8E)-4,4,8-trimethyl-7-oxocycloundeca-1,5,8-trien-1-yl]methyl acetate*. LC-MS (ESI) *m/z* (calculated for $C_{17}H_{25}O_3$) = 277.18 $[M+H]^+$ *m/z* (found) = 277.24 $[M+H]^+$, 299.22 $[M+Na]^+$. Deprotection of the acetate group was then performed using aqueous sodium hydroxide. Briefly, an aqueous solution of sodium hydroxide (1.9mL, 6.51mg, 0.163mmols) was added to the acetate (30mg, 0.108mmols) and the reaction mixture was stirred at room temperature for 6 hrs. The reaction mixture was then extracted with diethyl ether (3 X 6mL), concentrated and purified by flash column chromatography on silica gel to get the zerumbone alcohol, *(2E,6Z,10E)-6-(hydroxymethyl)-2,9,9-trimethylcycloundeca-2,6,10-trien-1-one* (**2**), as an off-white solid (22mg). ^1H NMR (400 MHz, $CDCl_3$) δ ppm 1.09 (s, 3H),

1.23 (s, 3H), 1.80 (s, 3H), 2.21-2.71 (m, 6H), 2.81 (br, 1H), 3.90 (s, 1H), 4.35 (s, 1H), 5.41 (t, J = 7.8Hz, 1H), 5.91 (d, J = 16.2Hz, 1H), 6.01 (d, J = 16.4Hz, 1H), 6.11 (t, J = 6.2Hz, 1H); LC-MS (ESI) *m/z* (calculated for $C_{15}H_{23}O_2$) = 235.17 $[M+H]^+$ *m/z* (found) = 235.35 $[M+H]^+$, 257.32 $[M+Na]^+$

(1*Z*,5*E*,8*E*)-4,4,8-trimethyl-7-oxo-*N*-(prop-2-yn-1-yl)cycloundeca-1,5,8-triene-1-carboxamide (**Yn-Zer**)

Attempted direct oxidation of the alcohol **2** to the corresponding acid using TEMPO/BAIB oxidation stopped at the intermediate aldehyde, *(1Z,5E,8E)-4,4,8-trimethyl-7-oxocycloundeca-1,5,8-triene-1-carbaldehyde*, which was subsequently oxidised to the acid using Pinnick oxidation. Briefly, to a solution of TEMPO (7.4mg, 0.047mmols) and BAIB (152mg, 0.47mmols) in a 1:1 (v/v) mixture of acetonitrile and water (0.75mL each) was added a solution of compound **2** in a 1:1 (v/v) mixture of acetonitrile and water (0.75mL each). The reaction mixture was stirred at room temperature for 16 hours. LC-MS analysis of the crude reaction mixture showed complete conversion of the starting material to the zerumbone aldehyde derivative whereas no carboxylic acid derivative was detected. The crude reaction mixture was diluted with dichloromethane (15mL) and extracted with water (2 X 10mL). The organic layer was concentrated and a quick purification was performed by flash chromatography on a short silica gel column to isolate the aldehyde (~10mg) from the reagents, characterized by LC-MS (ESI) *m/z* (calculated for $C_{15}H_{21}O_2$) = 233.15 $[M+H]^+$ *m/z* (found) = 233.26 $[M+H]^+$, 255.24 $[M+Na]^+$, and subjected directly for the Pinnick oxidation. Briefly, to a solution of the zerumbone aldehyde derivative in a 1:1 (v/v) mixture of t-BuOH and water (0.56mL each) was added 2-methyl-2-butene (50µL, 0.471mmols) followed by sodium chlorite (11.6mg, 0.129mmols) and monosodium phosphate (26.1mg, 0.218mmols). The reaction mixture was stirred at room temperature for 6 hrs upon which complete conversion of the aldehyde to the acid was observed by LC-MS. The crude reaction mixture was diluted with ethyl acetate (15mL) and extracted with water (2 X 10mL). The organic layer was concentrated and partially purified by flash chromatography on a short silica gel column to get the zerumbone acid derivative, (1*Z*,5*E*,8*E*)-4,4,8-trimethyl-7-oxocycloundeca-1,5,8-triene-1-carboxylic acid, as an off-white solid (10.6mg) which was used in the next step without further purification. LC-MS (ESI) *m/z* (calculated for $C_{15}H_{21}O_3$) = 249.15 $[M+H]^+$ *m/z* (found) = 249.32 $[M+H]^+$, 271.30 $[M+Na]^+$. The alkyne derivative of zerumbone (Yn-Zer) was synthesized by HATU-mediated coupling of the acid with propargylamine. Briefly, to a solution of the zerumbone acid in 0.6mL DMF was added N,N-diisopropylethylamine (11.3µL, 0.065mmols) and HATU (16.35mg, 0.043mmols) with stirring. After 5 min, propargylamine (3.34µL, 0.052mmols) was added and stirring continued for 1 hour. The reaction was then diluted with dichloromethane (15mL) and extracted water (2 X 10mL). The organic layer was concentrated and purified by flash column chromatography on silica gel to get the target probe Yn-Zer as a pale yellow solid (11mg, 23% over 6

steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 1.10 (s, 3H), 1.24 (s, 3H), 1.86 (s, 3H), 2.28 (t, J = 2.6Hz, 1H), 2.18-2.72 (m, 6H), 4.12 (s, 2H), 5.52 (t, J = 8Hz, 1H), 5.71 (s, 1H), 5.91 (t, J = 6Hz, 1H), 6.02 (d, J = 16.5Hz, 1H), 6.35 (d, J = 16Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 12.41, 25.56, 28.93, 35.50, 37.17, 43.72, 71.81, 79.24, 127.61, 133.00, 136.65, 139.49, 147.24, 161.26, 169.07, 204.41. HRMS: m/z calculated for $C_{18}H_{24}NO_2$ 286.1807 found 286.1842.

2. 3. Cell culture, preparation of whole cell lysates and lysate-based labelling

Cell culture media and reagents were obtained from Sigma Aldrich and Gibco (Life technologies). HeLa cells were grown in DMEM supplemented with 10% FBS without antibiotics in a humidified atmosphere with 10% CO_2 at 37^{o}C. The cells, at about 80-90% confluency, were washed with 1X PBS and trypsinised. Cell pellets were obtained by centrifugation at 1000 r.p.m at 4^{o}C for 5 min and the pellets were washed three times with 1X PBS. To prepare whole-cell lysate, the cell pellets were first suspended in a hypotonic buffer (10 mM HEPES, pH 7.5, 2 mM $MgCl_2$, 0.1% tween-20, 20% glycerol, Roche Complete EDTA-free protease inhibitors) and incubated for 10 min at 4℃. The suspension was centrifuged at 16,000*g* for 15 min at 4℃ and the supernatant was separated from the pellet. The pellets was then resuspended in a high-salt buffer (50 mM HEPES, pH 7.5, 420 mM NaCl, 2 mM $MgCl_2$, 0.1% tween-20, 20% glycerol, Roche Complete EDTA-free protease inhibitors) and incubated for 30 min at 4℃. The suspension was centrifuged at 16,000*g* for 15 min at 4℃ and the supernatant was collected. It was then combined with the soluble fractions in the hypotonic buffer to get the whole cell lysate. After protein quantification using Bradford assay (Bio-Rad DC™ Protein Assay), the lysate was subjected to labelling using Yn-Zer. Briefly, 100µg lysate at 1µg/µL concentration was treated with Yn-Zer, or DMSO as a control, in the presence, or absence of excess of zerumbone or α-humulene for 2 hrs at room temperature. The lysates were then subjected to CuAAC using the trifunctional capture reagent AzTB for 1.5 hrs. The click reactions were quenched by adding 10mM EDTA and proteins were precipitated using $CHCl_3$-MeOH-Water system. The precipitated protein pellets were washed with MeOH (x 2) and air-dried. The pellets were then solubilized in PBS with 2% SDS and 10mM DTT to 10mg/mL and diluted finally to 1mg/mL using 1X PBS. Proteins were resolved on 12% SDS gels using PAGE and the gels were scanned for fluorescence using an Ettan™ DIGE Imager (GE Healthcare).

2. 4. Intact cell-based labelling

HeLa adherent cells were grown in DMEM supplemented with 10% FBS without antibiotics in a humidified atmosphere with 10% CO_2 at 37^{o}C. The cells, at about 80-90% confluency, were washed twice with 1X PBS and incubated in fresh DMEM-10% FBS media with zerumbone/α-humulene/DMSO as a control for 30 min followed by treatment with Yn-Zer for 2 hrs. The cells were

then washed three times with 1X PBS and whole-cell lysates were prepared, quantified and subjected to CuAAC using AzTB as described above. After protein precipitation and re-solubilisation, labelling was visualized using in-gel fluorescence scanning following SDS-PAGE as described above.

2. 5. Spike-in-SILAC workflow and proteomics

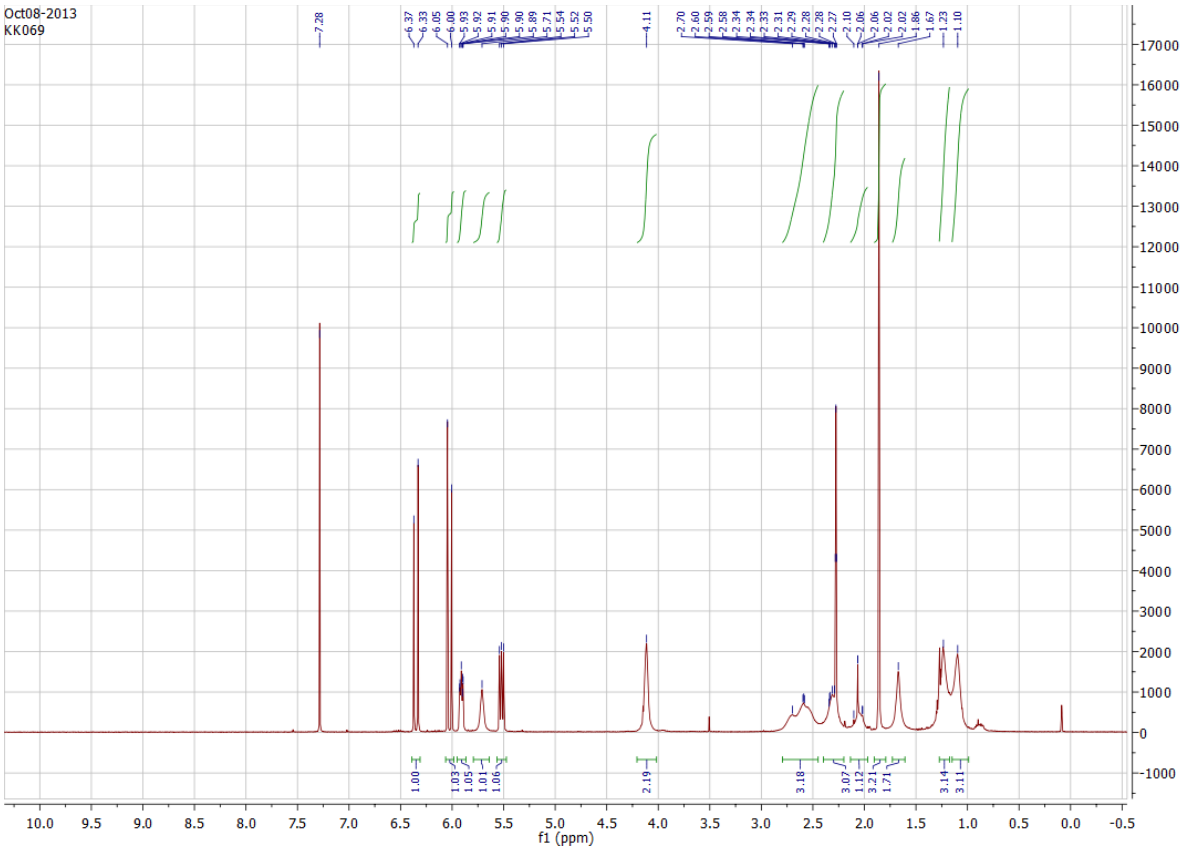
HeLa cells cultured in DMEM media (with 10% FBS) were treated separately in triplicate with DMSO alone or with three different concentrations (75, 100 and 150µM respectively) of zerumbone for 30 min at 37^{o}C in a humidified atmosphere with 10% CO_2. In parallel, HeLa cells labelled with $^{15}N_4{}^{13}C_6$-arginine and $^{15}N_2{}^{13}C_6$-lysine (termed R10K8 HeLa cells or 'heavy' HeLa cells) cultured and maintained in R10K8 DMEM media (with 10% dialysed FBS) were treated with DMSO for the same period. The 'light' and 'heavy' cells were then treated with 20µM of Yn-Zer for 2 hrs. After compound feeding and incubation, the cells were lysed and the lysates were quantified using DC^{TM} Protein Assay (Bio-Rad). Lysates from the R10K8 cells served the 'spike-in'SILAC standard. Heavy and light lysates were then mixed in 1:1 ratio (300µg total protein from each sample to yield 600µg total protein per final sample mix) and subjected to click reaction using the trifunctional capture reagent AzTB as described above. The samples, after protein precipitation to remove excess of the click reagents, were re-dissolved in PBS with 2% SDS and 10mM DTT to 10mg/mL and diluted finally to 1mg/mL using 1X PBS and subjected to affinity purification on NeutrAvidin-Agarose beads (1.5 hrs incubation on a rotating shaker at r.t.). The beads after extensive washings (x 4 with 0.5% SDS in 1X PBS, x 2 with 4M Urea in 1X PBS and x 5 with 50mM freshly prepared and filtered ammonium bicarbonate) were subjected to on-bead sequential reduction using DTT (30 min incubation at 50^{o}C followed by washing (x 2) with 50mM freshly prepared and filtered ammonium bicarbonate), alkylation using iodoacetamide (30 min incubation at r.t in dark followed by washing (x 2) with 50mM freshly prepared and filtered ammonium bicarbonate) and tryptic digestion (16 hrs at 37^{o}C in a shaking incubator). The digests were desalted on StageTips (using C_{18} Empore disks from Sigma Aldrich) and the desalted peptide mixtures were evaporated to dryness on a speed vac. The dried peptide mixtures were redissolved in a 0.5%/2%/97.5% (v/v/v) TFA/Acetonitrile/Water mixture with sonication. The samples were then subjected to centrifugation at 17,000*g* for 10 min at 10℃ and the clear solutions obtained were subjected to nanoLC-MS/MS on a Q Exactive (Thermo Scientific) proteomic mass spectrometer (Electrospray ionisation with EasyNano source and EasyNano columns for proteomics). The raw files from the mass-spectrometer were processed for protein identification and quantification using MaxQuant software[4] (version 1.4.1.2) and the data were analysed and visualized using Persues (version 1.4.0.6).

Ratios heavy/light (H/L), corresponding to the amount of protein in the lysate with increasing amount of the parent compound for each protein, each condition (concentration of zerumbone) and each replicate (n = 3 biological replicates) were obtained from MaxQuant. The data was filtered to remove

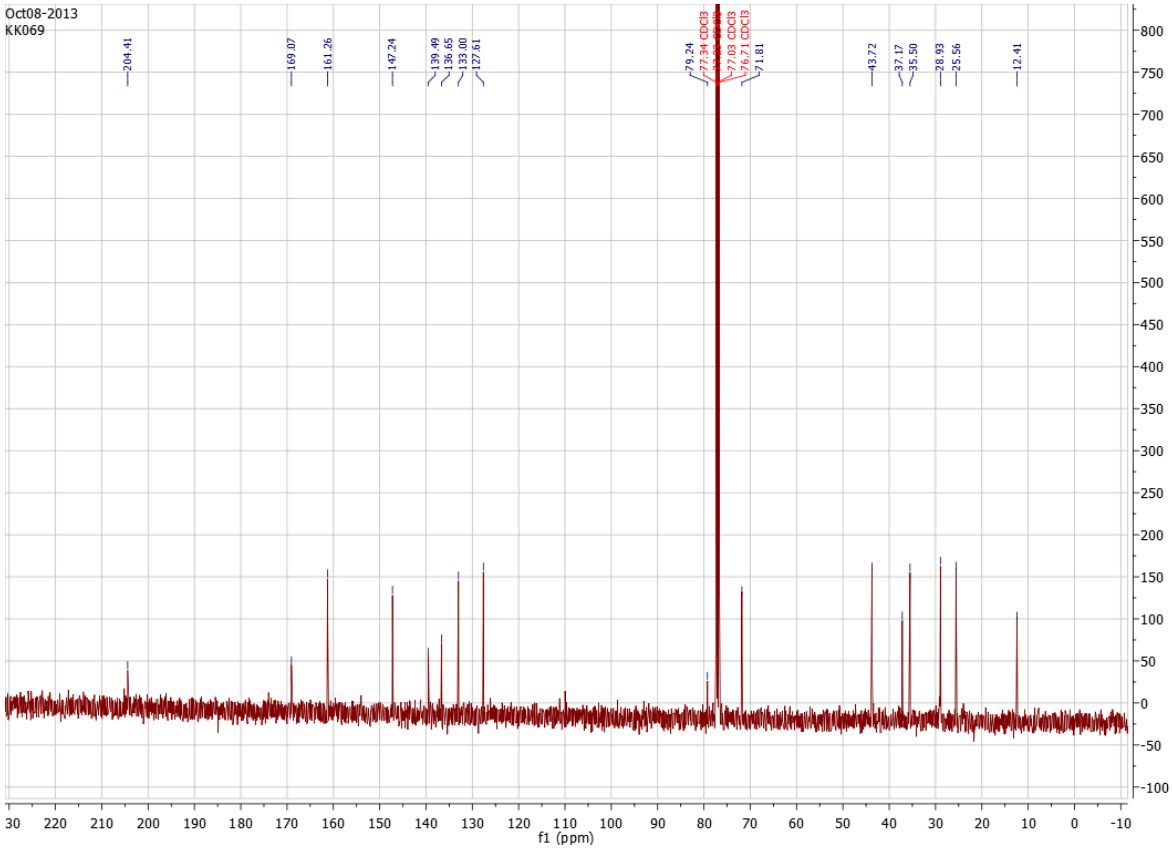
contaminant proteins. The data was further filtered to require 6 valid values per protein across the 12 samples. Ratio of ratios (H/L ratio of sample without zerumbone treatment over H/L ratio for each concentration of zerumbone treatment) were then determined for each concentration of zerumbone treatment. In order to compare the four conditions (four concentrations of zerumbone treatment including the no zerumbone treatment), an analysis of variance (ANOVA) was performed in Perseus with threshold P value of 0.05 for each protein target identified. In order to narrow down the target list to identify the most significantly engaged targets, the variance of the means of the H/L ratios of each protein corresponding to each tested concentration of zerumbone across the three biological replicates was calculated in Perseus with a threshold p value of 0.05 and the ANOVA significance (-log ANOVA p values) was plotted as a function of log2 fold change in the H/L ratios.

3. NMR Spectra

^{1}H NMR spectrum of Yn-Zer



^{13}C NMR spectrum of Yn-Zer



4. References

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