Electronic Supplementary Material (ESI) for Chemical Communications.

In cell Scalaradial Interactome Profiling Using a Bio-Orthogonal Clickable Probe

C. Cassiano,[a] L. Margarucci, [a] R. Esposito, [a] R. Riccio, [a]A. Tosco, [a]A. Casapullo, [a] * M.C. Monti [a] *

[a]Department of Pharmacy, University of Salerno, Via Giovanni Paolo II 132, 84084, Fisciano, Salerno, Italy email: mcmonti@unisa.it, casapullo@unisa.it tel: 0039-089-969243 fax: 0039-089-966202.

Supporting Information

SLD reaction with 11-azido-3,6,9-trioxaundecan-1-amine (TRX-Az) linker:

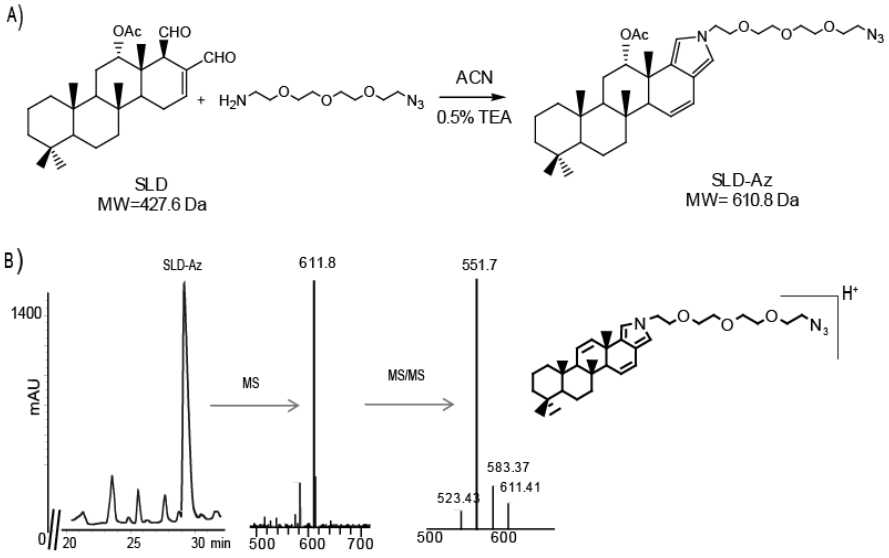


Figure S1. Panel A: Reaction between SLD and TRX-Az. Panel B: HPLC-UV trace, MS and MS/MS spectra of the reaction main product. In the MS spectrum, the base peak, at *m/z* of 611.8, corresponds to the protonated SLD-Az adduct, while its fragment at *m/z* of 551.7, in MS/MS spectrum, is generated by the loss of acetic acid.

Cu(I) catalyzed cyclization of SLD-azide with 4-pentyn-1-amine (PINA linker):

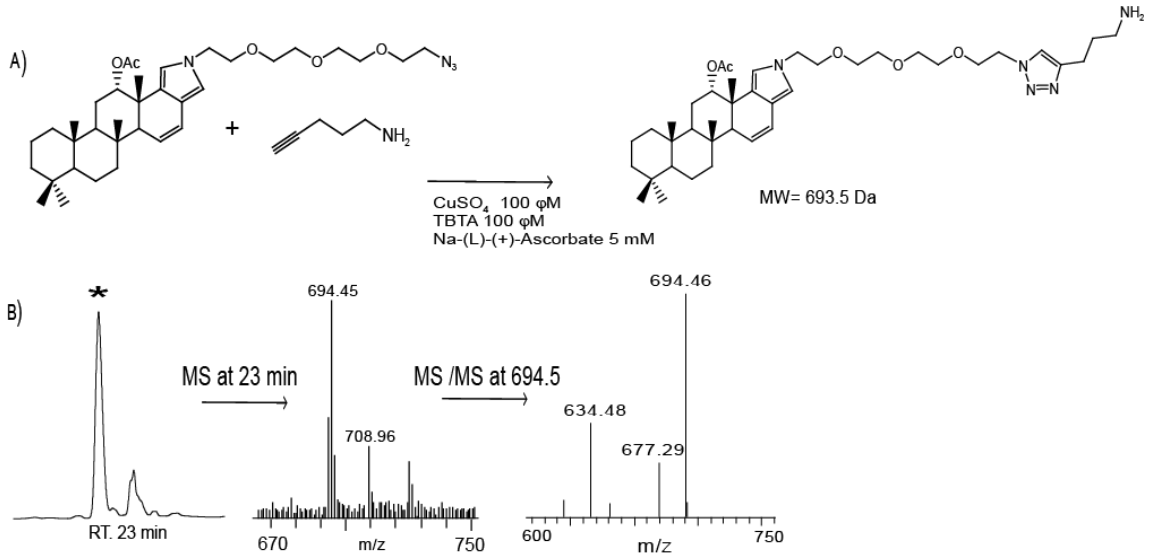


Figure S2. Panel A: SLD-Az reaction with the linker PINA. Panel B: HPLC-UV trace, MS and MS/MS spectra of the main product. In the MS spectrum, the base peak at *m/z* of 694.5 corresponds to the protonated click-chemistry adduct between SLD-Az and PINA, while its fragments at *m/z* of 677.3 and 634.5, in the MS/MS spectrum, descend from the loss of ammonia and acetic acid, respectively.

SLD reaction with the 2-(2-amino-ethyl-disulfanyl)-ethyl-amine (TIOS) linker, CDI agarose matrix modification and *in vitro* affinity purification of bee venom PLA_2:

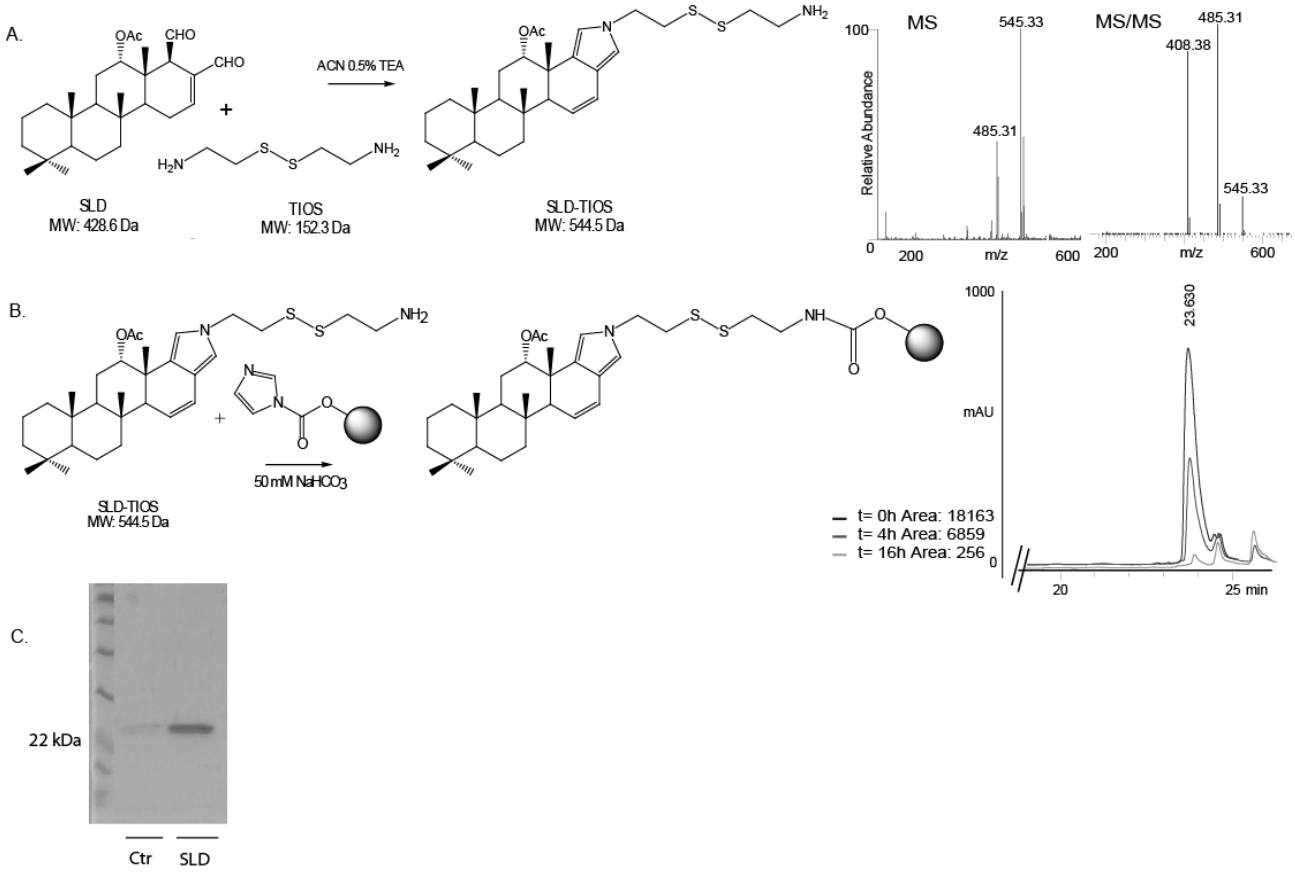
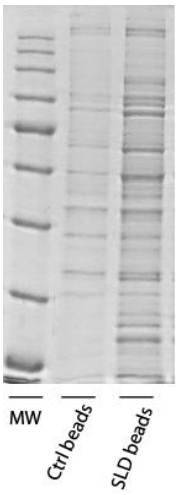


Figure S3. Panel A: SLD reaction with TIOS and MS and MS/MS spectra. In the MS spectra, the base peak at *m/z* 545.3 corresponds to the protonated SLD-TIOS adduct, while its fragment at m/z of 485.3 descends from the spontaneous loss of acetic acid. MS/MS spectra also show an additional peak at *m/z* 408.3,descending from the loss of 2-aminoethanethiol. Panel B: SLD-TIOS immobilization on CDI agarose solid matrix. HPLC-UV trace at 220 nm of the reaction supernatant after 0,4,16 h of incubation. Panel C: *In vitro* fishing for partners of SLD immobilized on CDI resin on a sample containing bovine serum albumin (66 kDa), lysozyme (14 kDa) and bee venom PLA_2 (around 20 kDa). SLD is able to fish out bee venom PLA_2 which is its main known partner.

In vitro SLD interacting targets:

A crude extract sample of HeLa cells was incubated for 1h at 4°C with an aliquot of the SLD-bearing matrix to allow the interactions between the compound and its potential macromolecular partners. The amount of un-specifically interacting proteins was reduced by several washing steps of the matrix beads, while the tightly bound proteins were released after the disulphide bond cleavage with DTT and then resolved by 12% SDS-PAGE (Fig. S4). In order to filter the specific and residual non-specific interactions, a control experiment was performed using the beads bearing the acetylated linker. The gel lanes were divided in few pieces and subjected to an *in situ* digestion protocol. The peptide mixtures were analyzed through nano-flow RP-HPLC MS/MS and the proteins identification was performed by submitting the peak lists to the Mascot database. The list of the SLD interacting proteins was refined by removing the hits shared with the control experiments.



n.	SwissProt	Proteins	Matches
1	PRDX1_HUMAN	Peroxiredoxin-1	22
2	EF1A1_HUMAN	Elongation factor 1-alpha 1	19
3	1433E_HUMAN	14-3-3 protein epsilon	18
4	1433B_HUMAN	14-3-3 protein beta/alpha	16
5	1433T_HUMAN	14-3-3 protein theta	14
6	1433Z_HUMAN	14-3-3 protein zeta/delta	12
7	1433G_HUMAN	14-3-3 protein gamma	11
8	1433F_HUMAN	14-3-3 protein eta	11
9	PRDX2_HUMAN	Peroxiredoxin-2	10
10	CYBP_HUMAN	Calcyclin-binding protein	9
11	ECH1_HUMAN	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial	9
12	PRDX6_HUMAN	Peroxiredoxin-6	8
13	PRDX4_HUMAN	Peroxiredoxin-4	8
14	GBLP_HUMAN	Guanine nucleotide-binding protein subunit beta-2-like 1	7
15	MARE1_HUMAN	Microtubule-associated protein RP/EB family member 1	7
16	ANXA5_HUMAN	Annexin A5	6
17	RS3_HUMAN	40S ribosomal protein S3	5
18	MDHM_HUMAN	Malate dehydrogenase, mitochondrial	4
19	EF1B_HUMAN	Elongation factor 1-beta	4
20	CPNS1_HUMAN	Calpain small subunit 1	4
21	RS8_HUMAN	40S ribosomal protein S8	3
22	CN166_HUMAN	UPF0568 protein C14orf166	3
23	PSA5_HUMAN	Proteasome subunit alpha type-5	3
24	ANXA1_HUMAN	Annexin A1	3
25	PSMG1_HUMAN	Proteasome assembly chaperone 1	3
26	ECHM_HUMAN	Enoyl-CoA hydratase, mitochondrial	2
27	EF1D_HUMAN	Elongation factor 1-delta	2
28	LYPA1_HUMAN	Acyl-protein thioesterase 1	2
29	PSME1_HUMAN	Proteasome activator complex subunit 1	2
30	PSME3_HUMAN	Proteasome activator complex subunit 3	2
31	HSPB1_HUMAN	Heat shock protein beta-1	2
32	CLIC1_HUMAN	Chloride intracellular channel protein 1	2
33	PSA4_HUMAN	Proteasome subunit alpha type-4	2
34	EI2BA_HUMAN	Translation initiation factor eIF-2B subunit alpha	2

Figure S4. 12 % SDS-PAGE of proteins eluted from *in vitro* fishing for partners of SLD and control pull-down. The list of proteins has been refined removing the common hits with the control experiments. Their best MASCOT identification of 3 independent experiments is reported with matched peptides.

Comparison between SLD and SLD-Az biological properties

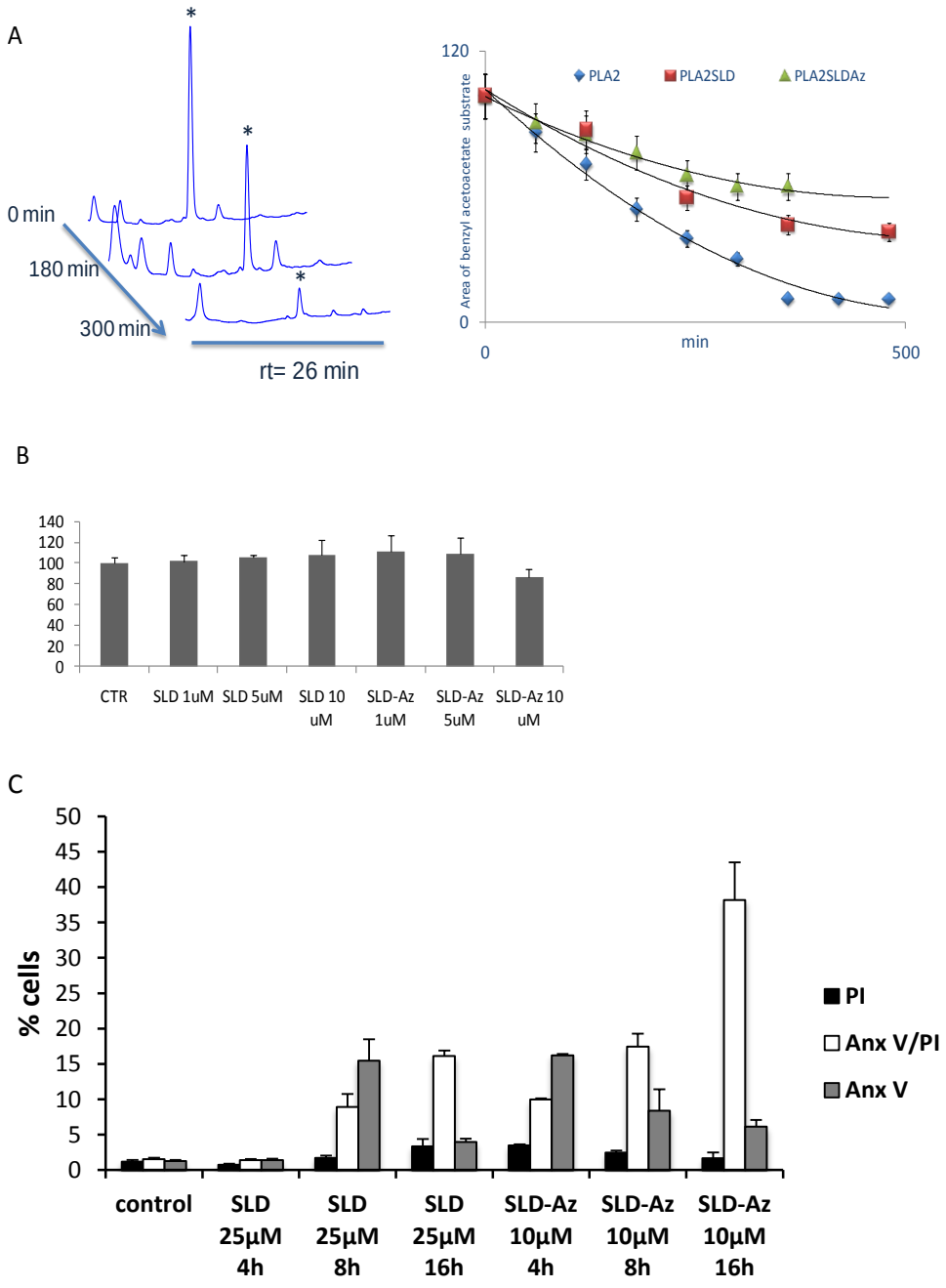
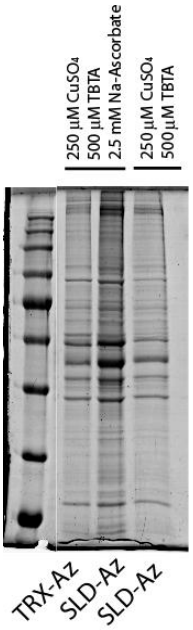


Figure S5. Panel A: RP-HPLC trace at 220 nm of the peak relative to benzyl acetoacetate (rt 26 min) which is selectively hydrolyzed by bee venom PLA_2 over time. The same experiment has been performed in presence of 10 fold molar excess of SLD and SLD-Az (over PLA_2 at 10 µM). The substrate area has been plotted over incubation time and SLD and SLD-Az inhibition curve are reported in the graph. Panel B: MTT analysis shows that both SLD and SLD-Az does not affect HeLa cell proliferation at tested doses. Panel C: Apoptosis analysis by Annexin V/PI staining shows that both molecules are able to induce apoptotic cell death, even if SLD-Az is effective at lower times and concentrations than SLD.

In cell SLD interacting targets:



n.	SwissProt	Proteins	Matches	n.	SwissProt	Proteins	Matches
1	FLNC_HUMAN	Filamin-C	104	46	CSN2_HUMAN	COP9 signalosome complex subunit 2	21
2	H13_HUMAN	Histone H1.3	80	47	HGS_HUMAN	epatocyte growth factor-regulated tyrosine kinase substrat	20
3	PARP1_HUMAN	Poly [ADP-ribose] polymerase 1	59	48	SYFA_HUMAN	Phenylalanine--tRNA ligase alpha subunit	20
4	U520_HUMAN	U5 small nuclear ribonucleoprotein 200 kDa helicase	57	49	SEPT2_HUMAN	Septin-2	19
5	EIF3A_HUMAN	Eukaryotic translation initiation factor 3 subunit A	53	50	LDHB_HUMAN	L-lactate dehydrogenase B chain	19
6	CAND1_HUMAN	Cullin-associated NEDD8-dissociated protein 1	53	51	TPM4_HUMAN	Tropomyosin alpha-4 chain	19
7	XPO2_HUMAN	Exportin-2	51	52	RAB1B_HUMAN	Ras-related protein Rab-1B	18
8	NSUN2_HUMAN	tRNA (cytosine(34)-C(5))-methyltransferase	50	53	RAN_HUMAN	GTP-binding nuclear protein Ran	18
9	GFPT1_HUMAN	Glutamine--fructose-6-phosphate aminotransferase [isomerizing] 1	41	54	TGM2_HUMAN	Protein-glutamine gamma-glutamyltransferase 2	18
10	SYQ_HUMAN	Glutamine--tRNA ligase	41	55	H2B1M_HUMAN	Histone H2B type 1-M	17
11	CTNA1_HUMAN	Catenin alpha-1	40	56	RL21_HUMAN	60S ribosomal protein L21	17
12	CND3_HUMAN	Condensin complex subunit 3	39	57	ARMC6_HUMAN	Armadillo repeat-containing protein 6	17
13	DDX1_HUMAN	ATP-dependent RNA helicase DDX1	36	58	KPRA_HUMAN	hosphoribosyl pyrophosphate synthase-associated protein	17
14	ACLY_HUMAN	ATP-citrate synthase	36	59	PRI1_HUMAN	DNA primase small subunit	17
15	LA_HUMAN	Lupus La protein	35	60	SUGT1_HUMAN	Suppressor of G2 allele of SKP1 homolog	16
16	RFC4_HUMAN	Replication factor C subunit 4	35	61	RCD1_HUMAN	Cell differentiation protein RCD1 homolog	16
17	IPO4_HUMAN	Importin-4	33	62	ARFG1_HUMAN	ADP-ribosylation factor GTPase-activating protein 1	15
18	IPO5_HUMAN	Importin-5	32	63	FEN1_HUMAN	Flap endonuclease 1	15
19	GALK1_HUMAN	Galactokinase	32	64	HAT1_HUMAN	Histone acetyltransferase type B catalytic subunit	14
20	1433F_HUMAN	14-3-3 protein eta	31	65	ARF3_HUMAN	ADP-ribosylation factor 3	14
21	IDH3A_HUMAN	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	30	66	INPP_HUMAN	Inositol polyphosphate 1-phosphatase	14
22	RL26_HUMAN	60S ribosomal protein L26	30	67	AN32B_HUMAN	idic leucine-rich nuclear phosphoprotein 32 family member	14
23	CN37_HUMAN	2',3'-cyclic-nucleotide 3'-phosphodiesterase	29	68	IMA7_HUMAN	Importin subunit alpha-7	13
24	1433B_HUMAN	14-3-3 protein beta/alpha	28	69	PSA5_HUMAN	Proteasome subunit alpha type-5	13
25	HNRH2_HUMAN	Heterogeneous nuclear ribonucleoprotein H2	27	70	LAT1_HUMAN	Large neutral amino acids transporter small subunit 1	13
26	PLIN3_HUMAN	Perilipin-3	26	71	UBC12_HUMAN	NEDD8-conjugating enzyme Ubc12	13
27	EHD4_HUMAN	EH domain-containing protein 4	26	72	PRDX4_HUMAN	Peroxiredoxin-4	12
28	RAB7A_HUMAN	Ras-related protein Rab-7a	25	73	PSB5_HUMAN	Proteasome subunit beta type-5	12
29	FA98B_HUMAN	Protein FAM98B	25	74	RLA2_HUMAN	60S acidic ribosomal protein P2	11
30	PRS6B_HUMAN	26S protease regulatory subunit 6B	24	75	RAB6A_HUMAN	Ras-related protein Rab-6A	11
31	ACTZ_HUMAN	Alpha-centractin	24	76	IF5A1_HUMAN	Eukaryotic translation initiation factor 5A-1	10
32	ESYT1_HUMAN	Extended synaptotagmin-1	24	77	ITPA_HUMAN	Inosine triphosphate pyrophosphatase	10
33	IMA1_HUMAN	Importin subunit alpha-1	23	78	SQSTM_HUMAN	Sequestosome-1	10
34	PSA7_HUMAN	Proteasome subunit alpha type-7	22	79	PURB_HUMAN	Transcriptional activator protein Pur-beta	10
35	PRS4_HUMAN	26S protease regulatory subunit 4	22	80	RFC2_HUMAN	Replication factor C subunit 2	10
36	GLRX3_HUMAN	Glutaredoxin-3	22	81	SAE1_HUMAN	SUMO-activating enzyme subunit 1	8
37	PRS8_HUMAN	26S protease regulatory subunit 8	22	82	TAGL2_HUMAN	Transgelin-2	8
38	TNPO3_HUMAN	Transportin-3	22	83	RL37A_HUMAN	60S ribosomal protein L37a	8
39	RAB8A_HUMAN	Ras-related protein Rab-8A	21	84	RBMS1_HUMAN	RNA-binding motif, single-stranded-interacting protein 1	8
40	PRS6A_HUMAN	26S protease regulatory subunit 6A	21	85	RL38_HUMAN	60S ribosomal protein L38	6
41	PROF1_HUMAN	Profilin-1	21	86	PRDX1_HUMAN	Peroxiredoxin-1	6
42	PRS7_HUMAN	26S protease regulatory subunit 7	21	87	F127C_HUMAN	Protein FAM127C	6
43	UBQL4_HUMAN	Ubiquilin-4	21	88	NDRG1_HUMAN	Protein NDRG1	6
44	DDX17_HUMAN	Probable ATP-dependent RNA helicase DDX17	21	89	ML12A_HUMAN	Myosin regulatory light chain 12A	5
45	PP2AB_HUMAN	Serine/threonine-protein phosphatase 2A catalytic subunit beta isoform	21	90	PRDX2_HUMAN	Peroxiredoxin-2	5

Figure S6. 12% SDS-PAGE of proteins eluted from *in cell* fishing for partners of SLD and control pull-down. The list of proteins has been refined removing the common hits with the control experiments. Their best MASCOT identification of 3 independent experiments is reported with matched peptides.

Material and Methods

Design and Synthesis of SLD-AZ:

SLD (1.5 mM) was coupled with 11-azido-3,6,9-trioxaundecan-1-amine (TRX-AZ, 15 mM) in ACN at 0.5% TEA for 1h at 25°C under stirring. Product formation has been monitored by RP-HPLC-UV at 220 nm using Agilent 1100 binary pump and Phenomenex column (Jupiter C18 150X2 mm 5 μ) at 0.2 mL/min. Gradient was from 5% to 95% buffer B (A= 100% H_2O and 0.1% TFA and B= 95% ACN, 5% H_2O and 0.1% TFA) in 25 min. Mass spectra were acquired on LCQ-DECA ThermoFinnigan equipped with an ESI source.

Click Chemistry Optimization:

SLD-Az (1.25 mM) has been incubated with the linker 4-pentyn-1-amine (PINA linker 2.50 mM) in PBS at pH 7.4 for 1h and less than 10% DMSO with 100 μM $CuSO_4$, 5 mM Na-L-(+)-Ascorbate and 100 μM Tris [(1-benzil-1,2,3-triazol-4-il)metal]amine (TBTA) under nitrogen and stirring. Product formation has been monitored by RP-HPLC-UV and MS as reported above.
500 μL of CDI resin have been mixed with 5 μmol of PINA linker in PBS buffer at pH of 7.4 for 3h at r.t.

In-Vitro Proteome Profiling:

SLD (0.6 mM) has been linked to a 2-(2-amino-ethyl-disulfanyl)-ethyl-amine linker (TIOS 6 mM) in ACN at 0.5% TEA for 30 min at r.t. Product formation has been monitored by RP-HPLC-UV at 220 nm using Agilent 1100 binary pump and Phenomenex column (Jupiter C18 150X2 mm 5 μ) at 0.2 mL/min. Gradient was from 5% to 95% buffer B (A= 100% H_2O and 0.1% TFA and B= 95% ACN, 5% H_2O and 0.1% TFA) in 20 min. Mass spectra were acquired on LCQ-DECA Thermo Finnigan equipped with an ESI source. SLD-TIOS (2.7 μmol) has been immobilized on 400 μL of CDI agarose in a final volume of 800 μL of ACN/$NaHCO_3$ 50 mM for 16 h under stirring at room temperature. HPLC runs were carried out as reported above at 4 different times as 0, 2, 4, 16 h. Control resin has been prepared using 400 μL of CDI CDI agarose in ACN/$NaHCO_3$ 50 mM using 0.7 μmol of TIOS linker alone. Free amine active groups on the resins were quenched by acetic anhydride 50 mM at r.t. A Kaiser test has been performed to verify the absence of free amines on modified resins.

A sample of bovine serum albumin, lysozyme and bee venom PLA_2 (all from Sigma-Aldrich) at concentration of 2 μM were mixed with SLD-bound beads suspension (50 μL) and the same amount of the control unbound matrix under continuous shaking (1 h, 4°C). The beads were collected by centrifugation (865*g*, 1 min, 4°C) and washed three times with PBS (pH 7.4). The bound proteins were eluted by boiling the beads in SDS-PAGE sample buffer (60 mMTris/HCl pH 6.8, 2% SDS, 0.001% bromophenol blue, 10% glycerol, 2% 2-mercaptoethanol). The eluted proteins were separated on 10% SDS-PAGE, and stained with Coomassie G-250 (Bio-Rad, Hercules, CA).

HeLa cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 U/mlpenicillin, 100 mg/ml streptomycin, at 37°C in a 5% CO_2 atmosphere (all reagents were from Euroclone). Cells were collected by centrifugation (600*g*, 5 min), washed twice with PBS and re-suspended in 1X ice cooled PBS containing Igepal (0.1%), supplemented with a protease inhibitor cocktail, and lysed by Dounce manual homogenization. Cellular debris were removed by centrifugation at 10000*g* for 10 min at 4 °C. Protein concentration was determined using Bradford assay and adjusted to 1 mg/ml. SLD-bound beads suspension (50 μL) and the same amount of the control

unbound matrix were separately incubated with 1 mg of HeLa total protein extract under continuous shaking (1 h, 4°C). The beads were collected by centrifugation (865*g*, 1 min, 4°C) and washed six times with PBS (pH 7.4). The bound proteins were eluted by boiling the beads in SDS-PAGE sample buffer (60 mMTris/HCl pH 6.8, 2% SDS, 0.001% bromophenol blue, 10% glycerol, 2% 2-mercaptoethanol). The eluted proteins were separated on 12% SDS-PAGE, and stained with Coomassie G-250 (Bio-Rad, Hercules, CA). SDS-PAGE gel lanes were cut and digested. The experiment has been repeated twice using an opportune control matrix bearing the linker without any metabolite. Each piece was washed with ultrapure water and CH_3CN and subjected to in situ protein digestion as described by Shevchenko.[27] Briefly, each slice was reduced with 10 mM 1,4-dithiothreitol (DTT) and alkylated with 54 mM iodoacetamide, then washed and rehydrated in trypsin solution (12 ng/μL) on ice for 1 h. After the addition of ammonium bicarbonate (30 μL, 50 mM, pH 7.5), proteins digestion was allowed to proceed overnight at 37°C. The supernatants were collected and peptides were extracted from the slices using 100% CH_3CN and both supernatants were combined. The peptide samples were dried and dissolved in formic acid (FA, 10%) before MS analysis. The peptide mixtures (5 μL) were injected into a nano-ACQUITY UPLC system (Waters). Peptides were separated on a 1.7 mm BEH C18 column (Waters) at a flow rate of 400 nL/min. Peptide elution was achieved with a linear gradient (solution A: 95 % H_2O,5 % CH_3CN, 0.1% FA; solution B: 95 % CH_3CN, 5 % H_2O, 0.1 % FA); 15–50% B over 55 min). MS and MS/MS data were acquired on a LTQ Orbitrap XL mass spectrometry system (Thermo Scientific). The ten most intense doubly and triply charged peptide ions were chosen and fragmented. The resulting MS data were processed by Mass Matrix MS data file conversion tool to generate mgf files for protein identifications. Database searches were carried out on the Mascot server (http://www.matrixscience.com/). The SwissProt database (release 2012_03, 21 March 2012, 535248 sequence entries, 189901164 amino acids abstracted from 208076 references) was employed (settings: two missed cleavages; carbamidomethyl (C) as fixed modification and oxidation (M) and phosphorylation (ST) as variable modifications; peptide tolerance 25 ppm; MS/MS tolerance 0.8 Da).

Later on, eluted proteins were separated on 12% SDS-page and transferred to a nitrocellulose membrane. The membrane was incubated for 1 h in a blocking solution containing 25 mM Tris pH 8, 125 mM NaCl, 0.05% Tween-20, 5% non-fat dried milk prior of exposition to primary antibodies raised against 14-3-3 ε (1:500) and PRX1 (1: 500; Novus Biologicals) overnight, at 4°C. Then, membrane was incubated for 1 h with an anti-rabbit peroxidase-conjugated secondary antibody (1:5000) (Sigma-Aldrich). 14-3-3- ε and PRX1 were detected by a chemo-luminescent detection system.

14-3-3 ε and PRX1 were immobilized onto two different flow cells of a CM5 sensor chip using standard amine coupling procedures. Phosphate–buffered saline, which consisted of 10 mM Na_2HPO_4 and 150 mM NaCl, pH 7.4, was used as running buffer. The carboxymethyl dextran surface was activated as already described and both proteins were diluted to a final concentration of 30 ng/μl in 10 mM sodium acetate, pH 4.5 and injected separately onto the two flow cells at a flow rate of 5 μl/min. After protein injections an increase of ≈9000 RU for PRX1 and ≈14000 RU for 14-3-3 was reached. The activated carboxymethyl dextran surface was finally blocked with a 7-min injection of 1.0 M ethanolamine-HCl, pH 8.5, at 5 μl/min. SLD solutions (0.01-10 μM), were prepared in running buffer containing 1% of DMSO and injected at least three times. Since they dissociated back to baseline within a reasonable time frame, no regeneration was required. The interaction experiments were carried out at a flow rate of 10 μl/min, employing a 3 min injection time. The dissociation time was set at 600 seconds. Rate constants for associations (k_a) dissociations (k_d) and the dissociation constants (K_D) were obtained by globally

fitting data from injections of all concentrations, using the BIAevaluation software, using the simple 1:1 Langmuir binding model.

In-Situ Proteome Profiling: SLD-AZ (1 and 10 μM) was incubated in HeLa cell lysate (obtained as described above) for 1 h at 4°C and then mixed with 50 μL of CDI-Agarose matrix bearing PINA linker in presence of $CuSO_4$, Na-L-(+)-Ascorbate and TBTA under nitrogen for 45 min at room temperature. As control, the experiment has been carried out in the same conditions using TRX-Az as bait. After several washes in PBS buffer to remove the unbound proteins, SLD partners were eluted, separated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was incubated for 1 h in a blocking solution containing 25 mM Tris pH 8, 125 mM NaCl, 0.1% Tween-20, 5% nonfat dried milk prior to overnight incubation, at 4°C, with a primary monoclonal antibody raised against 14-3-3 ε (1:500) and PRX1 (1: 500). Then, membrane was incubated for 1 h with an anti-rabbit peroxidase-conjugated secondary antibody (1:5000). 14-3-3- ε and PRX1 were detected by a chemo-luminescent detection system.

Biological evaluation of SLD and SLD-Az:

A solution of bee venom PLA_2 at 10 μM has been incubated with or without SLD and SLD-Az, separately, at 100 μM for 20 min at 37 °C under stirring and then benzyl-acetoacetate has been added at final concentration of 500 μM in PBS buffer containing $CaCl_2$. Aliquots of the mixture were injected on a RP-HPLC Agilent 1100 equipped with a C-8 proteo column (Phenomenex) at different times from 0 to 480 min of incubation. The area of the peak at 26 min (identified as benzyl-acetoacetate in control runs at λ of 220 nm) was measured.

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After SLD and SLD-Az treatments in a concentration range from 1 μM to 10 μM, the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan was used to assess the effect of the two molecules on cell viability.

Apoptosis was evaluated by Annexin V-PI staining kit (Clontech), according to the manufacturer instructions. Briefly, $1x10^5$ HeLa cells were seeded in 12-well plates before SLD and SLD-Az administration for 4, 8, and 16h. Then cells were harvested, stained for Annexin V-PI and analysed with a FACScan flow cytometer (Becton Dickinson).

Large-Scale Pull-Down/LC-MS/MS analysis: Living HeLa cells were growth as described above, treated with 1 μM of SLD-AZ and 1 μM of TRX-Az for 6 hours and then washed with fresh media to remove the excess of drug which did not pass through cell membranes. Cells were then lysed by PBS containing 0.1% IGEPAL and proteases inhibitor by Dounce manual homogenator. 1 mg of lysates were then treated with 250 μM $CuSO_4$, 2.5 mM Na-L-(+)-Ascorbate and 500 μM TBTA under nitrogen to promote Huisgen 1,3 cycloaddition on 10 μL of PINA derived CDI-Agarose beads for 45 min at room temperature. The same reaction was carried out both on the same sample without 2.5 mM Na-L-(+)-Ascorbate and on TRX-Az treated cell lysates as negative controls. Pull-down samples were then separated by SDS-PAGE followed both by immune-blotting analysis as described above or Comassie staining. SDS-PAGE gel lanes were cut and digested as reported above. Western blotting analysis on proteasome were carried out using primary monoclonal antibodies raised against α-7 (1:500) and Psmc3 (1: 500, Santa Cruz Biotecnology). Then, membrane was incubated for 1 h with an anti-mouse peroxidase-conjugated secondary antibody (1:5000). A chemo-luminescence detection system has been used.