

Supplementary Information

## **Chemical Proteomics-driven Discovery of Oleocanthal as Hsp90 Inhibitor**

Luigi Margarucci<sup>a</sup>, Maria Chiara Monti<sup>a</sup>, Chiara Cassiano<sup>a</sup>, Matteo Mozzicafreddo<sup>b</sup>, Mauro Angeletti<sup>b</sup>, Raffaele Riccio<sup>a</sup>, Alessandra Tosco<sup>a\*</sup> and Agostino Casapullo<sup>a\*</sup>

<sup>a</sup>*Dipartimento di Farmacia, Università degli Studi di Salerno, Via Ponte don Melillo, 84084 Fisciano, Italy.*

<sup>b</sup>*Scuola di Bioscienze e Biotecnologie, Università degli Studi di Camerino, via Gentile III da Varano, 62032 Camerino, Italy.*

Fax: +39-089-969602

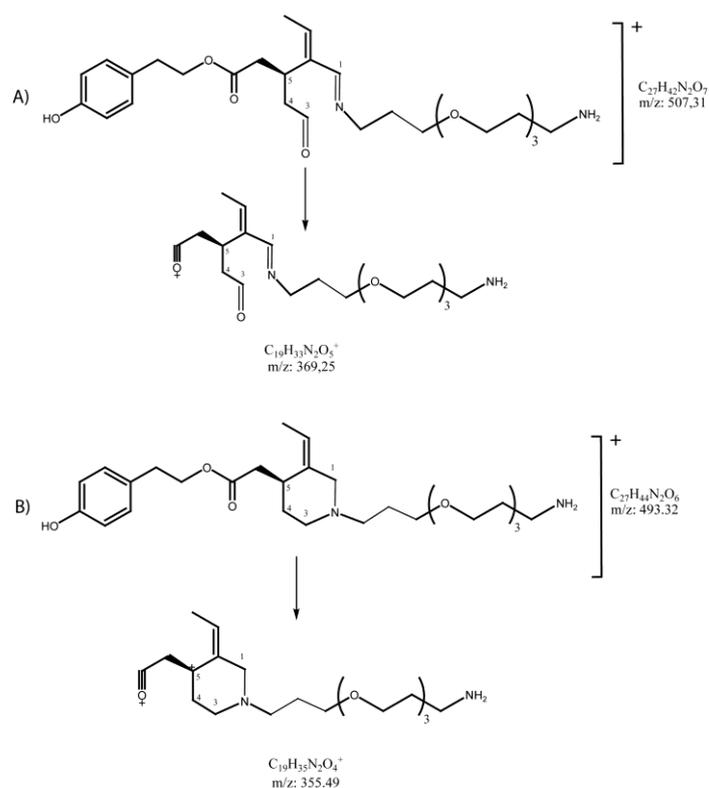
E-mail: [casapullo@unisa.it](mailto:casapullo@unisa.it), [tosco@unisa.it](mailto:tosco@unisa.it)

## SUPPLEMENTARY RESULTS

### S1. Tandem MS analysis of the covalent adduct OLC-4,7,10-trioxa-1,13-tridecanediamine

The reactive profile of OLC and 4,7,10-trioxa-1,13-tridecanediamine [NH<sub>2</sub>-(PEG)<sub>3</sub>-NH<sub>2</sub>], in presence and absence of the reducing agent NaBH<sub>4</sub>, was analyzed by LC-MS and ESI-MSMS. The reaction gave rise to a product with a m/z of 507.3, corresponding to the adduct produced upon the nucleophilic attack of the spacer terminal amino group onto the C-1 OLC aldehyde reported in **Figure S1**. The MS/MS fragmentation spectra of this ion gave rise to one daughter ion at m/z 369.2 as shown in **Figure S1**.

The same reaction was performed in presence of the reducing agent NaBH<sub>4</sub> giving rise to a cyclic adduct with a m/z of 493.2 Th. This peak, subjected to MSMS, generates the daughter ion at m/z 355.5 reported in **Figure S1**.



**Fig. S1.** Reaction products between OLC and 4,7,10-trioxa-1,13-tridecanediamine [NH<sub>2</sub>-(PEG)<sub>3</sub>-NH<sub>2</sub>] measured by ESI-MS. **A)** Ion fragments obtained during tandem MS experiments on the parent ion at 507.3 Th. **B)** Ion fragments obtained during tandem MS experiments on the parent ion at 493.4 Th.

## S2. HPLC runs of OLC immobilization reaction on amine modified beads.

The amount of immobilized OLC was estimated integrating the peaks of the free OLC species after HPLC separation. The strong reduction of the peak area demonstrates that OLC has been anchored to the amine modified beads

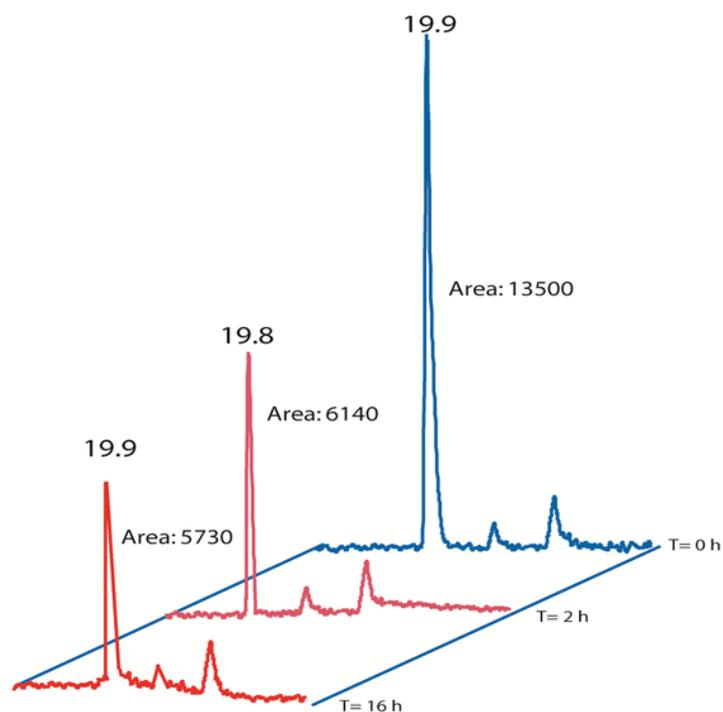
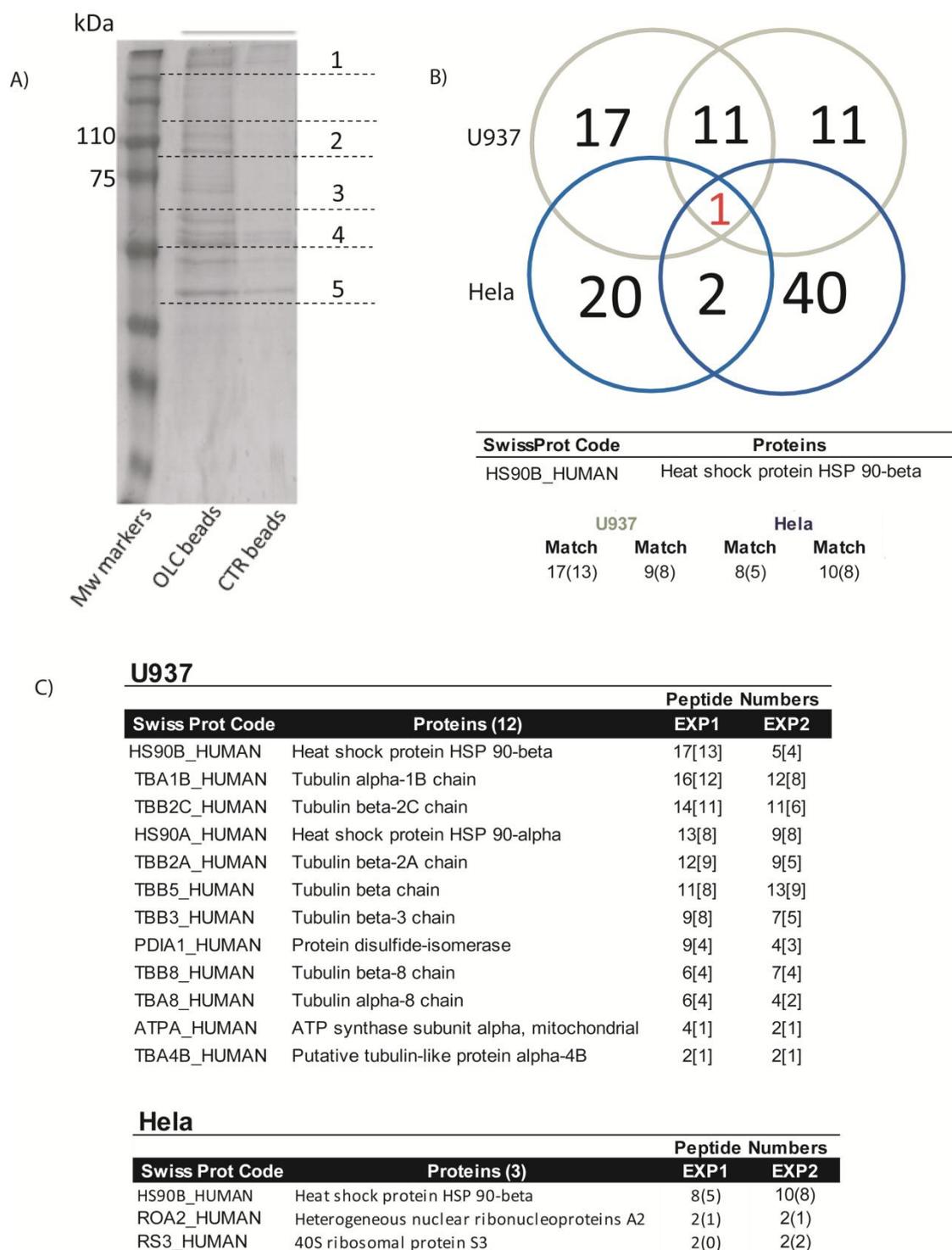


Fig. S2. The peak relative to free OLC at rt 19.9 min is shown after t=0, 2, 16 h of incubation ..

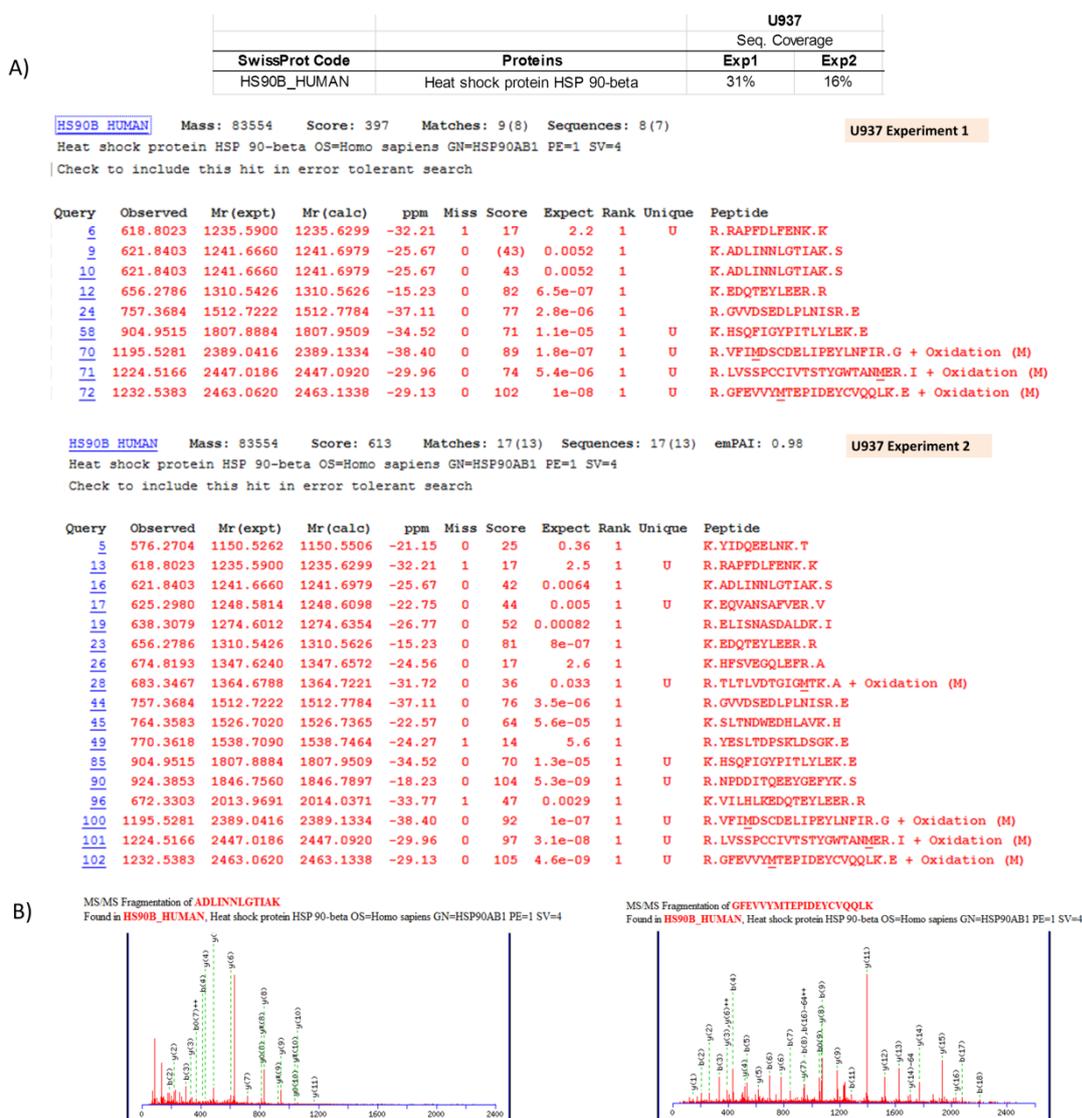
### S3. OLC interactors identification



**Fig. S3.** A) Representative gel of the affinity chromatography elutions reporting number and size of excised bands. B) Venn diagrams show the number of proteins identified after the *in situ* digestion of two independent chemical proteomics experiments with HeLa and U937 cell lysates (after deletion of proteins identified in control experiments). The overlapping of putative OLC partners in HeLa and U937 cell lysates leads to the identification of Hsp90 as the main interactor. C) The table reports identified putative OLC partners in HeLa and U937 cell lines.

## S4. Mascot report for Hsp90 and peak lists

Heat Shock protein beta fitting mass values of two independent experiments for both U937 and HeLa are reported herein. All the peak lists, which were sent to Mascot, are available in the pride database under the PRIDE experiment accession number 22688-22695 and the project title name “ChemProt\_OLC”<sup>[1]</sup>.



**Fig. S4.** Mascot report of all matched peptides of Hsp90 in two independent experiments in U937 cell lines. **A)** The table reports the sequence coverage of Hsp90 in U937 experiment and all matched peptides of Hsp90. **B)** MS/MS spectra of Hsp90 peptides ADLINNLGTIAK and GFEVVYMTPEIDYCVQQLK has been reported.

A)

		Hela	
		Seq. Coverage	
SwissProt Code	Proteins	Exp1	Exp2
HS90B_HUMAN	Heat shock protein HSP 90-beta	17%	13%

**HS90B\_HUMAN** Mass: 83554 Score: 157 Matches: 8(5) Sequences: 8(5) Hela Experiment 1

Heat shock protein HSP 90-beta OS=Homo sapiens GN=HSP90AB1 PE=1 SV=4  
 Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
<a href="#">5</a>	523.2662	1044.5178	1044.5927	-71.70	1	4	42	5	U	K.VEKVTISNR.L
<a href="#">12</a>	576.2716	1150.5286	1150.5506	-19.06	0	40	0.011	1	U	K.YIDQEELNK.T
<a href="#">15</a>	625.2889	1248.5632	1248.6098	-37.32	0	35	0.037	1	U	K.EQVANSAPVER.V
<a href="#">18</a>	638.3114	1274.6082	1274.6354	-21.28	0	50	0.0011	1	U	R.ELISNASDALDK.I
<a href="#">22</a>	656.2652	1310.5158	1310.5626	-35.68	0	16	2	1	U	K.EDQTEYLEER.R
<a href="#">37</a>	757.3557	1512.6968	1512.7784	-53.90	0	79	1.5e-06	1	U	R.GVDSSEDLPLNISR.E
<a href="#">54</a>	924.3861	1846.7576	1846.7897	-17.37	0	4	53	1	U	R.NPDDITQEYGEFYK.S
<a href="#">71</a>	1195.5320	2389.0494	2389.1334	-35.14	0	53	0.00073	1	U	R.VFIMDSCDELIPEYLNfir.G + Oxidation (M)

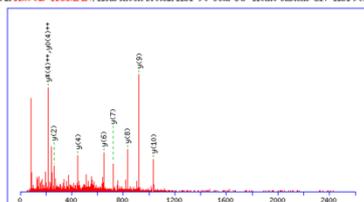
**HS90B\_HUMAN** Mass: 83554 Score: 269 Matches: 10(7) Sequences: 10(7) emPAI: 0.48 Hela Experiment 2

Heat shock protein HSP 90-beta OS=Homo sapiens GN=HSP90AB1 PE=1 SV=4  
 Check to include this hit in error tolerant search

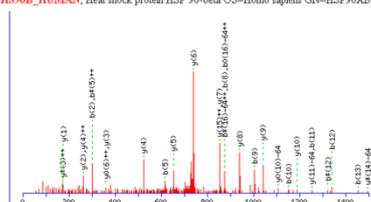
Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
<a href="#">63</a>	523.2931	1044.5716	1044.5927	-20.19	1	6	24	6	U	K.VEKVTISNR.L
<a href="#">102</a>	625.3267	1248.6388	1248.6098	23.2	0	42	0.0066	1	U	K.EQVANSAPVER.V
<a href="#">112</a>	638.3346	1274.6546	1274.6354	15.1	0	56	0.00029	1	U	R.ELISNASDALDK.I
<a href="#">127</a>	674.8536	1347.6926	1347.6572	26.3	0	20	1.2	1	U	K.HFSVGGLEFR.A
<a href="#">172</a>	757.4000	1512.7854	1512.7784	4.67	0	89	1.4e-07	1	U	R.GVDSSEDLPLNISR.E
<a href="#">176</a>	772.9097	1543.8048	1543.8205	-10.17	1	24	0.42	1	U	R.ELISNASDALDKIR.Y
<a href="#">243</a>	924.4130	1846.8114	1846.7897	11.8	0	43	0.0074	1	U	R.NPDDITQEYGEFYK.S
<a href="#">254</a>	672.3565	2014.0477	2014.0371	5.26	1	39	0.015	1	U	K.VILHLKEDQTEYLEER.R
<a href="#">262</a>	731.6573	2191.9501	2191.9328	7.89	0	80	1.4e-06	1	U	R.YHTSQSGDEMTSLSEYVSR.M + Oxidation (M)
<a href="#">274</a>	1195.5720	2389.1294	2389.1334	-1.65	0	86	3.8e-07	1	U	R.VFIMDSCDELIPEYLNfir.G + Oxidation (M)

B)

MS/MS Fragmentation of **ELISNASDALDK**  
 Found in **HS90B\_HUMAN**, Heat shock protein HSP 90-beta OS=Homo sapiens GN=HSP90AB1 PE=1 SV=4



MS/MS Fragmentation of **YHTSQSGDEMTSLSEYVSR**  
 Found in **HS90B\_HUMAN**, Heat shock protein HSP 90-beta OS=Homo sapiens GN=HSP90AB1 PE=1 SV=4



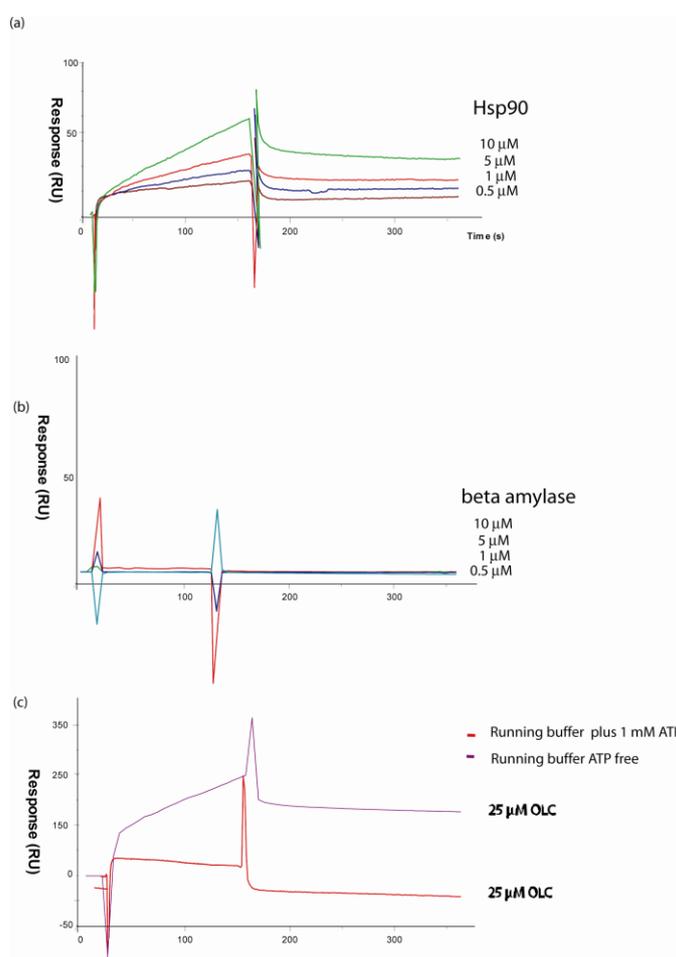
**Fig. S5.** Mascot report of all matched peptides of Hsp90 in two independent experiments in HeLa cell line. **A)** Table reports the sequence coverage of Hsp90 and all matched peptides of Hsp90. **B)** MSMS spectra of Hsp90 peptides ELISNASDALDK and YHTSQSGDEMTSLSEYVSR has been reported.

## S6. Analysis of OLC-Hsp90 binding by Surface Plasmon Resonance

Hsp90 was immobilized on a CM-5 sensor chip and OLC was injected in a concentration range from 0.5 to 10  $\mu\text{M}$ . Sensorgrams in **Figure S6** panel A clearly demonstrate the existence of a direct interaction between OLC and Hsp90, validating the chemical proteomics results.

OLC was injected over a  $\beta$ -amylase-immobilized sensor chip as negative control. As shown in Figure S6 B, no binding was detected at each OLC concentration.

Then, 1 mM ATP was added to the running buffer to block the nucleotide binding site and 25  $\mu\text{M}$  OLC was injected over the immobilized Hsp90. As reported in **Figure S6** panel C no binding was observed between the counterparts.

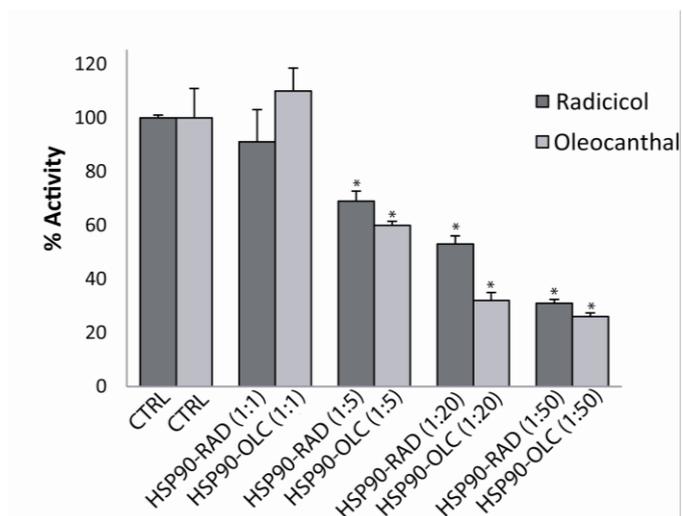


**Fig. S6.** a) Sensorgrams obtained from the binding of OLC (0.5-10  $\mu\text{M}$ ) to immobilized Hsp90.

b) Sensorgrams obtained from the injection of OLC (25  $\mu\text{M}$ ) to immobilized Hsp90 in absence and presence of 1mM ATP diluted in the running buffer.

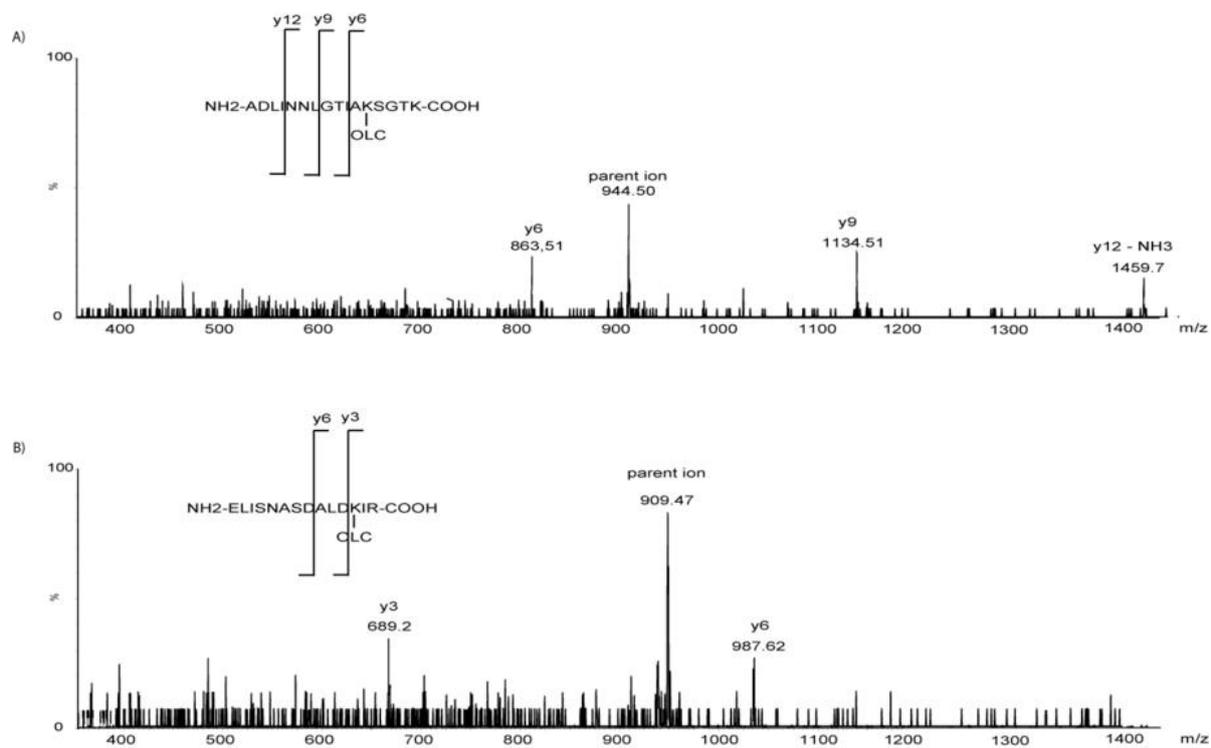
c) Sensorgrams obtained from the injection of OLC (0.5-10  $\mu\text{M}$ ) on immobilized  $\beta$ -amylase.

## S7. ATPase activity assay



**Fig. S7.** ATPase activity of human recombinant Hsp90 in presence of its co-chaperone Aha1 at different molar excesses of OLC and radicolol (used as positive control). All data were performed in triplicate and reported as percentage of the control. \*\* $p < 0.01$ .

## S8. Tandem mass spectra of Hsp90 peptides modified by OLC.



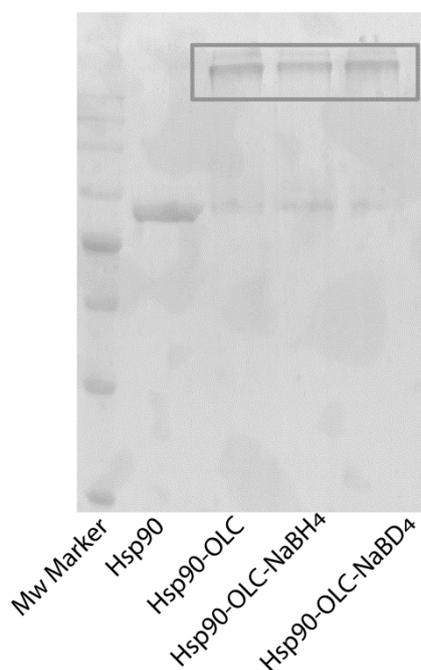
**Figure S8.** A) MSMS spectrum of the Hsp90 peptide (ADLNNLGTIAKSGTK) identifies K112 as the covalent binding site of OLC. B) MSMS spectrum of the Hsp90 peptide (ELISNASDALDKIR) identifies K58 as the covalent binding site of OLC.

### S9. Searching for putative cross-linking sites.

An extensive characterization of OLC-treated samples was carried out to discover the presence of covalently polymerized adduct through mass spectrometry, developing an assay using  $\text{NaBH}_4/\text{NaBD}_4$  labelling to detect OLC-bearing peptides by high resolution LCMSMS analysis.

As reported in Figure S9, we submitted two OLC-treated Hsp90 samples, separately treated with  $\text{NaBH}_4$  or  $\text{NaBD}_4$ , to SDS PAGE, along with the free Hsp90 as control. Then, the corresponding bands at 90 and around 300 kDa, were cut and subjected to trypsin digestion and high resolution LC-MSMS analysis.

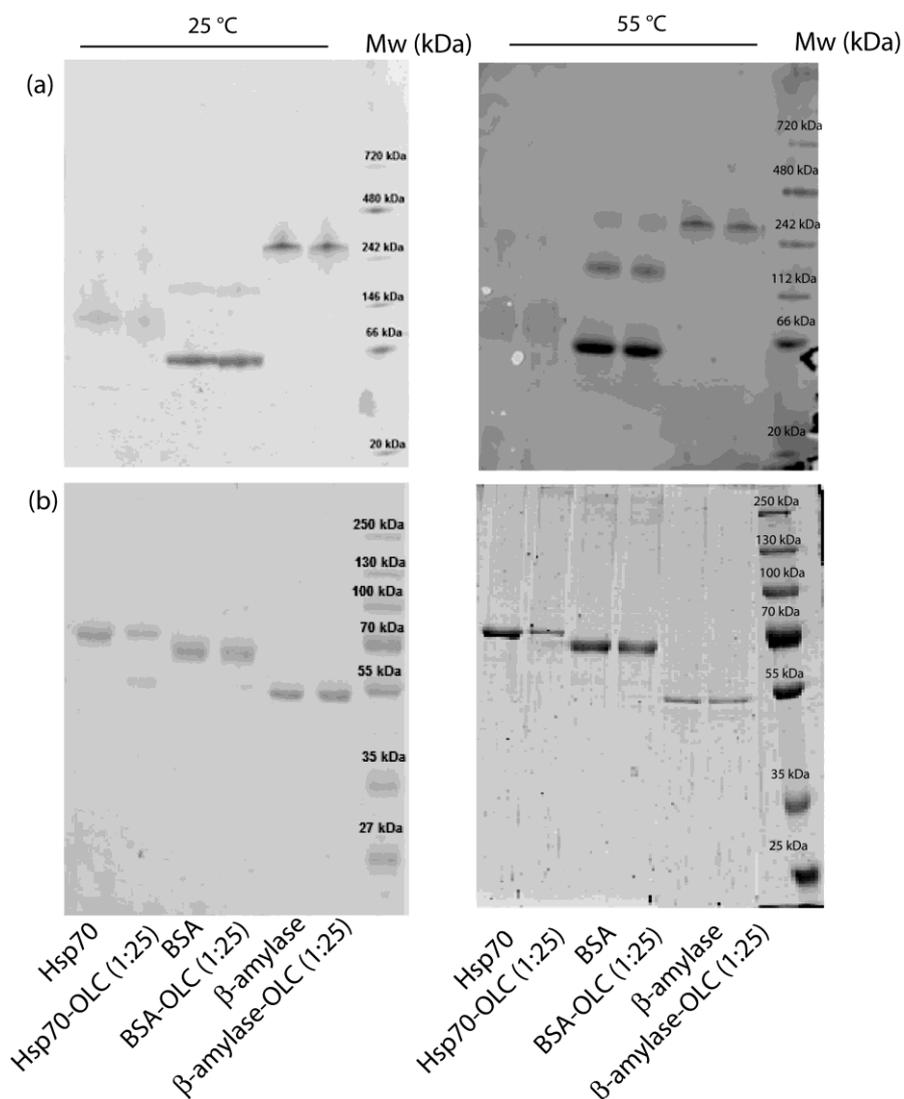
This procedure allows an easy MS detection of the OLC-modified peptides thanks to their different isotopic labelling. However, after comparison of the LCMSMS runs of  $\text{NaBH}_4$  and  $\text{NaBD}_4$  labelled samples, we were able to solely find single peptides containing Lys112 or Lys58 modified by OLC and no cross-linked species, that could account for a covalent nature of the high molecular weight oligomers. The experiment was repeated twice to confirm the data.



**Figure S9.** SDS-PAGE of 2  $\mu\text{g}$  of Hsp90 alone and treated with OLC (molar excess of 10 fold) and  $\text{NaBH}_4$  or  $\text{NaBD}_4$  (molar excess of 100 fold on Hsp90 for 45 min at  $0^\circ\text{C}$ ). The Comassie stained bands were cut and digested by trypsin. LCMSMS analysis on peptide mixtures were carried out as reported in the paper.

### S10. OLC selectively influences Hsp90 oligomerization

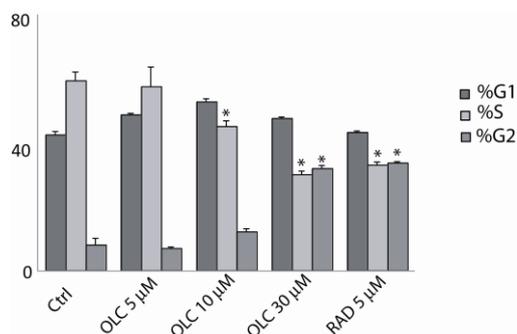
Hsp70, bovine serum albumin (BSA) and  $\beta$ -amylase were incubated with a tenfold molar excess of OLC for 10 min at 25 and 55 °C to assess the selective ability of OLC to influence Hsp90 oligomerization. As reported in the **Figure S10**, OLC did not modify proteins oligomerization, nor induce their aggregation.



**Figure S10. A)** Blue Native-PAGE of Hsp70, bovine serum albumin (BSA) and  $\beta$ -amylase in presence and absence of 10 fold molar excess of OLC. **B)** SDS PAGE of Hsp70, bovine serum albumin (BSA) and  $\beta$ -amylase in presence and absence of 10 fold molar excess of OLC.

## S11. Cell cycle analysis and viability assay

U937 cells were seeded in 24-well plastic plates and treated with OLC at different concentrations and with radicicol (AppliChem GmbH, Germany), as reference compound, at 5  $\mu\text{M}$ . After 24 h of treatment, quantitative analysis of cell cycle distribution by flow cytometry was performed (Figure S11).



**Fig. S11.** Cell cycle analysis of U937 at different concentrations of OLC. All the experiments were performed at least in triplicate.

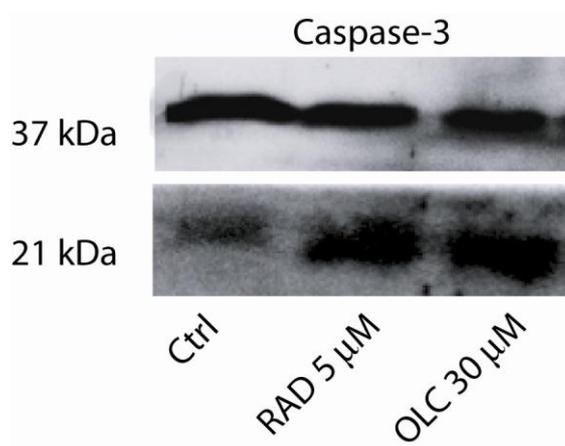
To shed light on the cytotoxic effect of OLC over normal cell lines, cell viability of PBMC (Peripheral Blood Mononuclear Cell) in comparison to U937 and HeLa cell lines was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells, seeded in 96-well microtiter plates, were exposed (for 24 and 48h) to different concentrations of OLC ranging from 10 to 100  $\mu\text{M}$  in media containing 0.1% DMSO. The mitochondrial-dependent reduction of MTT to formazan was used to assess cell viability. The experiments were carried out in quadruplicate, and all the values were expressed as percentage of the control containing 0.1% DMSO. As expected, oleocanthal induces only a slight reduction of viability. (Table S1)

	IC50 ( $\mu\text{M}$ )	
<b>U937</b>	OLC 24 h	46.6 $\pm$ 2,3
	OLC 48 h	25 $\pm$ 1,25
	RAD 24 h	20.2 $\pm$ 1,1
	RAD 48 h	1.5 $\pm$ 0.07
<b>Hela</b>	OLC 24 h	86.2 $\pm$ 4.3
	OLC 48 h	88.9 $\pm$ 4.4
	RAD 24 h	33.8 $\pm$ 1.6
	RAD 48 h	16.7 $\pm$ 0.8
<b>PBMC</b>	OLC 24 h	>100
	OLC 48 h	>100
	RAD 24 h	>100
	RAD 48 h	>100

**Table S1.** IC50 values of OLC in two cancer cell lines along with normal cell lines are reported herein. Each value is the mean  $\pm$  standard deviation of three independent experiments.

### S12. OLC pro-apoptotic activity

To investigate OLC pro-apoptotic activity we performed a western blot analysis on caspase 3 from U937 cells treated with 30  $\mu\text{M}$  oleocanthal or 5  $\mu\text{M}$  radicicol for 24h, observing in both cases increasing levels of the cleaved caspase 3.



**Figure S12.** Western blot analysis of caspase 3 in U937 cells incubated with OLC 30  $\mu\text{M}$  and radicicol 5  $\mu\text{M}$  for 24h

## EXPERIMENTAL SECTION

### Production of OLC functional matrix

21  $\mu\text{mol}$  of 4,7,10-trioxa-1,13-tridecanediamine ( $\text{NH}_2\text{-(PEG)}_3\text{-NH}_2$ ) dissolved in  $\text{NaHCO}_3$  (50 mM) with 30%  $\text{CH}_3\text{CN}$  at pH 8.5 were added to 0.6 ml (dry volume) of the CDI (Carbonyldiimidazol) activated Reacti-Gel (6X) beads and incubated for 16 h at  $50^\circ\text{C}$  under continuous shaking.

After three washings with ethanol, beads were subjected to Kaiser test in order to prove the presence of free amine groups on the modified Reacti-Gel surface. The obtained beads were then treated with 16.4  $\mu\text{mol}$  of OLC (5 mg) dissolved in 400  $\mu\text{l}$  of  $\text{NaHCO}_3$  (10 mM) and ACN 30% at pH 8.5 for 12 h, and the sample was treated with  $\text{NaBH}_4$  100 mM in sodium borate buffer (10 mM) at pH 8.5 for 30 min. The amount of immobilized OLC was estimated integrating the peaks of the free OLC species after HPLC injections of supernatants at  $t=0$  h,  $t=2$  h and  $t=12$  h, using an Agilent 1100 Series chromatographer. HPLC runs were carried out using a Phenomenex C18 column (250 x 2.0 mm) at a flow rate of 200  $\mu\text{l}/\text{min}$ . The gradient (Solution A: 0.1% TFA, solution B: 0.07% TFA, 5%  $\text{H}_2\text{O}$  95%  $\text{CH}_3\text{CN}$ ) started at 10% and ended at 95% B sol. after 25 min.

Modified matrix was treated with 300 mM acetic anhydride dissolved in DMF at 10% TEA. Similar experimental conditions were applied for preparation of the control matrix. Briefly, 0.6 ml of activated Reacti-Gel (6X) support were incubated with 21  $\mu\text{mol}$  of 4,7,10-trioxa-1,13-tridecanediamine ( $\text{NH}_2\text{-(PEG)}_3\text{-NH}_2$ ) as described, and the sample was treated with acetic anhydride. The matrices were stored at  $4^\circ\text{C}$ .

### Affinity purification of OLC partners

HeLa and U937 cell lines were grown in DMEM medium (Sigma) supplemented with 10% (v/v) foetal bovine serum (Sigma), 100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin (Sigma), 4 mM glutamine (Sigma), 10 mM HEPES (Sigma), 10 mM sodium pyruvate (Sigma) at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  atmosphere. For cell lysates, cells were collected by centrifugation, washed twice with PBS 1X and re-suspended in lysis buffer (PBS 1X, 0.1 % Igepal) supplemented with protease inhibitor cocktail. The obtained suspensions were sonicated for 2 min with Vibracell (Sonics) setting an amplitude of 30% and cellular debris were removed by centrifugation at 10000 x g for 10 min at  $4^\circ\text{C}$ . Protein concentration was determined using Bradford assay and adjusted to 1 mg/ml.

50  $\mu\text{l}$  of OLC beads suspension and the same amount of the control matrix were separately incubated with 1 mg of HeLa (or U937) total proteins under continuous shaking for 16 h at  $4^\circ\text{C}$ . Beads were collected by centrifugation (1000 x g, 3 min,  $4^\circ\text{C}$ ) and washed six time with PBS. The bound proteins were eluted by boiling the beads in 30  $\mu\text{l}$  of SDS-PAGE sample buffer (60 mM Tris/HCl pH 6.8, 2% SDS, 0.001%

bromophenol blue, 10% glycerol, 2% 2-mercaptoethanol). The eluted proteins were subjected to SDS-PAGE separation on a 10% polyacrylamide gel and stained with colloidal Coomassie (Biorad G-250).

### **OLC interactors identification**

The SDS-PAGE gel lanes from control and OLC based experiments were cut, washed with MilliQ Water and CH<sub>3</sub>CN and subjected to *in situ* protein digestion as described in Shevchenko protocol.<sup>[1]</sup> Briefly, gel slices were reduced and alkylated using 1,4-dithiothreitol (10 mM) and iodoacetamide (54 mM) respectively, then washed and rehydrated in trypsin solution (10 ng/μl) on ice for 1h. After the addition of 30 μl ammonium bicarbonate (10 mM, pH 7.5), samples were digested overnight at 37 °C. The supernatants were collected and peptides were extracted by the gel slices using 100% CH<sub>3</sub>CN. Samples were dried out and dissolved in 10% FA before MS analysis. 5 μl of the obtained peptide mixture were injected onto a nano Acquity LC system (Waters Corp. Manchester, United Kingdom). The peptides were separated using a 1.7 μm BEH C-18 column (Waters Corp. Manchester, United Kingdom) at a flow rate of 400 nl/min. Peptide elution was achieved with a linear gradient from 15 to 50% (solution A: 95% H<sub>2</sub>O, 5% CH<sub>3</sub>CN, 0.1% FA; solution B: 95% ACN, 5% H<sub>2</sub>O, 0.1% FA) in 55 min. MS and MS/MS data were acquired using a Q-TOF Premier mass spectrometer (Waters Corp., Micromass, Manchester, United Kingdom). Five most intense doubly and triply charged peptide-ions were automatically chosen by the MassLynx software and fragmented. After mass spectrometric measurements, data were automatically processed by ProteinLynx software to generate peak lists for protein identifications. Database searches were carried out with MASCOT server. The SwissProt database (release 2010\_11 of 02 Nov 10, 522019 sequences, 184241293 residues) was used, allowing 2 missed cleavages, carbamidomethyl (C) as fixed modification and oxidation (M) and phosphorylation (ST) as variable modifications. The peptide tolerance was set to 80 ppm and the MS/MS tolerance to 0.8 Da. All the peak lists sent to Mascot, are available in the pride database under the PRIDE<sup>[2]</sup> experiment accession number 22688-22695 and the project title name “ChemProt\_OLC”.

### **Western Blotting Analysis on OLC protein partners**

The protein mixtures fished out by control and OLC beads in U937 cell lysates were analyzed by Western blotting. 10 μl of each sample were resolved on a 10% 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 overnight, at 4°C, with a primary monoclonal antibody raised against Hsp90 at 1:2000 dilution (BD biosciences, cod. 610418). Then, membrane was incubated for 1 h with an anti-mouse peroxidase-conjugated secondary antibody (1:5000). Hsp90 was detected by a chemo-luminescence detection system.

### **OLC pull-down of human recombinant Hsp90**

20  $\mu\text{L}$  of immobilized OLC and control beads were incubated with 2  $\mu\text{g}$  of recombinant full-length Hsp90 $\beta$  (SPP-777, BD Biosciences) in 1 ml of PBS buffer for 1 h at 4  $^{\circ}\text{C}$ . The bound protein was eluted by boiling the beads in presence of SDS sample buffer. Samples were resolved on a 10% SDS-PAGE and stained with colloidal Coomassie (Biorad G-250) staining.

### **SPR analysis**

SPR analyses were performed on a Biacore 3000 optical biosensor equipped with a research-grade CM5 sensor chip. Using this platform, Hsp90 $\beta$  (SPP-777, BD Biosciences) and  $\beta$ -amylase (both at 30  $\mu\text{g}/\text{mL}$  in 10 mM sodium acetate, pH 4.5) were immobilized on two different flow cells at a flow rate of 5  $\mu\text{L}/\text{min}$  using standard amine-coupling protocols obtaining immobilization levels of around 13000 RU for each protein.

Oleocanthal was injected at different concentrations ranging from 0.1 to 10  $\mu\text{M}$  in running buffer (xx). Binding experiments were performed at 25  $^{\circ}\text{C}$ , using a flow rate of 30  $\mu\text{L}/\text{min}$ , with 180 s monitoring of association and 300 s monitoring of dissociation.

### **ATPase Assay**

A fluorescence ADP release assay was performed using ADP Hunter Plus Assay kit (DiscoverRx) accordingly to the manufacturer's instructions. 8  $\mu\text{L}$  of Hsp90 (3  $\mu\text{M}$ ) was incubated for 30 min at 28  $^{\circ}\text{C}$  at different molar excess of OLC (from 1:1 to 1:50) or radicicol. Then, incubation mixtures were diluted by adding 12  $\mu\text{L}$  of 15 mM HEPES, 20 mM NaCl, 1 mM EGTA, 0,02% Tween 20, 10 mM  $\text{MgCl}_2$ , 0,1  $\text{mg}\cdot\text{mL}^{-1}$  BGG (bovine  $\gamma$ -globulins) containing 1.2  $\mu\text{M}$  of Aha1 (StressMarq) and 500  $\mu\text{M}$  ATP. ADP release was measured using a Perkin Elmer LS 55 fluorimeter (530 nm excitation and 590 nm emission). All experiments were carried out in triplicate and reported as percentage of the control experiment in which ATPase activity of free Hsp90 was measured.

### ***In silico* analysis of the interaction between Hsp90 and OLC by molecular docking**

Molecular docking of OLC onto Hsp90 was performed on Pentium4/Linux Red Hat based platform using Autodock 4.0.<sup>[3]</sup> X-ray crystal structure of human Hsp90 catalytic domain (pdb ID: 1YET)<sup>[4]</sup> was obtained from the Protein Data Bank<sup>[5]</sup>.

After preparing receptor and ligand Autodock pdbqt files removing water and other ligands included in the pdb file and adding polar hydrogen atoms, OLC was docked onto Hsp90 using a grid of 70 $\times$ 70 $\times$ 70 points around the whole molecule and a grid of 60 $\times$ 60 $\times$ 54 points around the active site, and setting all the parameters as previously published.<sup>[6]</sup>

Then, Discover module of InsightII software was used to refine the most probable ligand/receptor complexes, minimizing all molecules, and Ludi module was used to calculate the complexes affinity, as previously reported<sup>[7]</sup>.

The output complexes, all modelling studies, and images were rendered with PyMOL (2010, DeLano Scientific LLC, San Carlos, CA). PyMOL was also used to calculate the predicted length of hydrogen bonds established in the complexes and the distances between atoms.

### **Cell cycle analysis**

For cell cycle analysis, 500  $\mu$ L of U937 cells ( $2.5 \times 10^5$  cells/mL) and  $3 \times 10^4$  of HeLa cells per well were seeded in 24-well plastic plates, and treated with OLC at different concentrations and with radicicol (AppliChem GmbH, Germany), as reference compound, at 5  $\mu$ M. After 24 h of treatment, 500  $\mu$ L of hypotonic buffer (33 mM sodium citrate, 0.1% Triton X-100, 50  $\mu$ g/mL propidium iodide) was added to cell suspensions. Cells were analyzed with a FACScan flow cytometer (Becton Dickinson, CA), using Mod FitLT program for quantitative analysis of cell cycle distribution. All the experiments were performed at least in triplicate.

### **Western blot analysis on Hsp90 client proteins**

OLC (50 mM) and the reference compound radicicol (50 mM) were freshly prepared dissolving powder in DMSO and following dilution in culture medium. Sixteen hours after seeding U937 (10 mL,  $2.5 \times 10^5$  cells/mL) cells were treated with OLC (30  $\mu$ M) and radicicol (5  $\mu$ M) separately for 24 h and then processed to obtain whole-cell extracts. Control experiments were performed by adding 0.2% DMSO to the medium. Each treatment was performed in triplicate. After incubations, cells were harvested by centrifugation; cell pellets were incubated with lysis buffer (PBS 1X, 0.1% Igepal supplemented with a protease inhibitors cocktail; Sigma Aldrich) for 30 min on ice.

To remove cellular debris, lysates were centrifuged for 10 min at 10000g at 4°C and protein concentration was determined by Bradford assay using bovine serum albumin (BSA) as standard.

Total protein extracts from U937 cells were resolved on a 10% SDS-PAGE and transferred onto a nitrocellulose membrane. Membranes were incubated for 1 h at RT in a blocking solution (25 mM Tris pH 8, 125 mM NaCl, 0.1% Tween- 20, 5% nonfat dried milk) and then over night at 4 °C with one of the subsequent antibodies: anti-Raf-1 (cod. 9422; Cell Signaling), anti-Cdk4 (cod. 559677; BD Biosciences), anti-Hsp90 (cod. 610418; BD Biosciences), anti-Akt, (cod. 9272; Cell Signaling), anti-Hsp70 (SPA-810; Stressgen), anti-caspase 3 (Sc-7148, Santa Cruz Biotechnology) and anti- $\beta$  actin (Sc-47778; Santa Cruz Biotechnology) for normalization, separately. Finally, membranes were incubated for 1 h with peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and proteins were detected with

a chemiluminescent substrate. Signals were captured by LAS4000 imaging system and quantitative analysis was performed using the ImageQuant software.

### Blue native gel electrophoresis

Blue native gel electrophoresis (BN-PAGE) was carried out using the Novex Bis-Tris system according to the manufacturer's specifications. Pre-cast NativePAGE™ Novex 4–16% (v/v) Bis-Tris gels were run with near neutral pH in presence of 0.4% (w/v) Coomassie blue G-250.

Each sample contained 3 µg of protein Hsp90β, Hsp70, β-amylase or BSA with or without a tenfold molar excess of OLC or radicicol. Samples were incubated for 10 min at 25 or 55°C and then separated for native and SDS PAGE analysis.

### Reference List

- [1] A. Shevchenko, H. Tomas, J. Havlis, J. V. Olsen, M. Mann, *Nat. Protoc.*, **2006**, *6*, 2856.
- [2] J.A. Vizcaíno, R. Côté, F. Reisinger, J.M. Foster, M. Mueller, J. Rameseder, H. Hermjakob, L. Martens, *Proteomics* **2009**, *18*, 4276-4283.
- [3] D.S. Goodsell, G.M. Morris, A.J. Olson, *J. Mol. Recognit.* **1996**, *9*, 1-5.
- [4] C.E. Stebbins, A.A. Russo, C. Schneider, N. Rosen, F.U. Hartl FU, N.P. Pavletich *Cell.* **1997**, *2*, 239-250.
- [5] H.M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T.N. Bhat, H. Weissig, I.N. Shindyalov, P.E. Bourne *Nucleic Acids Res.* **2000**, *28*, 235–242.
- [6] M. Cuccioloni, M. Mozzicafreddo, M. Spina, C.N. Tran, M. Falconi, A.M. Eleuteri AM, M. Angeletti M. *J Lipid Res.* **2011**, *52*, 897-907.
- [7] M. Mozzicafreddo, M. Cuccioloni, V. Cecarini, A. M. Eleuteri, and M. Angeletti. *J. Chem. Inf. Model.* **2009**, *49*, 401-409.