Electronic Supplementary Information

Liposome antibody-ionophore conjugate antiproliferative activity increases by cellular metallostasis alteration.

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EXPERIMENTAL SECTION

Chemicals. Protected N^{α}-Fmoc-amino acid derivatives, coupling reagents and Rink amide MBHA (4-methylbenzhydrylamine) resin were purchased from Calbiochem-Novabiochem (Laufelfingen, Switzerland). The Fmoc-21-amino-4,7,10,13,16,19-hexaoxaheneicosanoic acid, Fmoc-Ahoh-OH was purchased from Neosystem (Strasbourg, France). Egg phosphatidylcholine (PC) and cholesterol (Chol), were obtained from Avanti Polar Lipids, Alabaster. All other chemicals were commercially available by Sigma-Aldrich (Milan, Italy), Fluka (Bucks, Switzerland) or LabScan (Stillorgan, Dublin, Ireland) and were used as received unless otherwise stated. All solutions were prepared by weight with doubly distilled water. Preparative RP-HPLCs were carried out on a LC8 Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a UV Lambda-Max Model 481 LC detector using Phenomenex (Torrance, CA) C4 column. Elution solvents are H_2O/0.1% TFA (A) and CH_3CN/0.1% TFA (B), from 5% to 70% over 30 minutes at 20 mL/min flow rate. Purity and identity of the products were assessed by analytical LC-MS analyses by using Finnigan Surveyor MSQ single quadrupole electrospray ionization (Finnigan/Thermo Electron Corporation San Jose, CA), column: C4-

Phenomenex eluted with an H_2O/0.1% TFA (A) and CH_3CN/0.1% TFA (B) from 5% to 70% over 15 minutes at 0.8 mL/min flow rate.

Peptidic amphiphile (PA) synthesis. Peptide conjugates were synthesized by using standard solid-phase 9-fluorenylmethoxycarbonyl (Fmoc) procedures. The Rink amide MBHA resin (substitution 0.70 mmol/g) was used as the solid phase support to provide the peptides as C-terminus amide, and synthesis was performed on a scale of 0.2 mmol. The resin was swelled in DMF for 30 min and the Fmoc deprotection reaction (10 min) was performed twice by using 30% (v/v) piperidine in DMF. The coupling of amino acids was achieved by adding two-fold molar excess of amino acid, mixed with equimolar amounts of 1-hydroxybenzotriazole (HOBt), (benzotriazol-1-yloxy)-trispyrrolidinophosphonium hexafluorophosphate (PyBop) and four-fold molar excess of diisopropylethylamine (DIPEA) in DMF. All couplings were performed twice for 1 hour. The Fmoc ethoxylic spacer and lipophilic N,N-dioctadecylsuccinic acid were coupled, as previously described.[1]. Peptides were fully deprotected and removed from the resin with the TFA (trifluoroacetic acid)/TIS (triisopropylsilane)/H_2O (95.5/2.0/2.5 v/v/v) mixture at room temperature. Peptidic amphiphile was precipitated with ice-cold water, dissolved in H_2O/CH_3CN mixture and freeze-dried. The purification of the crude products was carried out by RP-HPLC. Mass spectra confirm the products identity. $(C_{18}H_{37})_2NCO(CH_2)_2CO$-$(AhOh)_2$-Ala-His-Amide; $(C18)_2$-L2-Car: Rt = 15.2 min; (MW = 1500.01) $[M + H]^+$ = 1500.98 amu and $[M + 2H]^+/2$ = 751.03 amu.

Preparation of aggregate solutions. All aggregate solutions were prepared by weight, buffering the samples at pH 7.4 by using 0.1 M phosphate buffer and checking the pH with a pH meter MeterLab PHM 220. The samples to be measured were mostly prepared from stock solutions. Pure micelles (5 mM) were prepared by dissolving the lyophilized $(C18)_2$-L2-Car in the buffer solution and passed through a 0.45 μm filter. Liposomal solutions of PC/Chol (60/40 molar ratio), PC/Chol/$(C18)_2$-L2-Car (60/35/5 molar ratio) and PC/Chol/$(C18)_2$-L2-Car/DSPE-Peg2000-Maleimide (58.5/35/5/1.5 molar ratio) were obtained by dissolving the required amounts of amphiphiles in 2 mL of a

methanol/chloroform (50/50 v/v) mixture. Subsequently the organic solvents were removed under a stream of nitrogen gas to obtain a homogeneous film on the wall of the vial. Any trace solvent was then removed keeping the vial under vacuum for 15 minutes. Then, the dry lipid film was hydrated with 2.0 mL of phosphate buffer and sonicated in an ultrasound bath for 30 minutes. The liposomal suspension (at a final concentration of 100 mM) was extruded 10 times at room temperature, using a thermobarrel extruder system (Northern Lipids Inc, Vancouver, BC, Canada) under nitrogen through a polycarbonate membrane (Nucleopore Track Membrane 25 mm, Whatman, Brentford, UK) having 0.1 μm pore size.

Fluorescence measurements. Values of critical micellar concentration (CMC) of $(C18)_2$-L2-Car was obtained by fluorescence measurements. Fluorescence spectra were recorded at room temperature on a Jasco Model FP-750 spectrofluorophotometer in a 1.0 cm path length quartz cell. Equal excitation and emission bandwidths were used throughout the experiments, with a recording speed of 125 nm min^{-1} and automatic selection of the time constant. The CMC were measured by using 8-anilino-1-naphthalene sulfonic acid ammonium salt (ANS) as the fluorescent probe.[2] Small aliquots of peptide aqueous solution were added to a fixed volume (1.00 mL) of $2.0 \cdot 10^{-5}$ M ANS fluorophore directly in the quartz cell. CMC values were determined by linear least-squares fitting of the fluorescence emission at 480 nm, upon excitation at 350 nm versus the amphiphile concentration.

Preparation of immunoliposomes

Mixed liposomes (PC/Chol/$(C18)_2$-L2-Car/DSPE-Peg2000-Maleimide at 58.5/35/5/1.5 molar ratio), were prepared as above described at 20 mM concentration. Anti-HER2-Fab' fragment (2.5 mg/mL), kindly provided by Dr. M. Ruvo of CNR-IBB of Naples, was incubated with a stoichiometric amount of TCEP (Tris(2-carboxyethyl)phosphine hydrochloride) reducing agent and the mixture was left under stirring for 20 minutes at room temperature. Successively, 0.1 equivalents of Anti-HER2-Fab', with respect to the maleimido function was added to the liposomal suspension under nitrogen atmosphere and reaction was left overnight. The unreacted Fab' were removed from the immunoliposomes by using Sepharose CL-4B column, pre-equilibrated with 0.1 M phosphate buffer.

Dynamic light scattering (DLS) characterization. Structural features of micelles, liposomes and immunoliposomes were obtained using DLS technique. DLS measurements were carried out using a Zetasizer Nano ZS (Malvern Instruments, Westborough, MA) that employs a 173° backscatter detector. Other instrumental settings were: measurement position 4.65 mm; attenuator 8; temperature 25 °C; cell, disposable sizing cuvettes. Samples of supramolecular aggregates were prepared at 2.0×10^{-4} M concentration and centrifuged at room temperature at 13,000 rpm for 5 min before use.

Serum stability. According to the procedure above reported, 10 mM mixed liposomes PC/Chol/$(C18)_2$-L2-Car were prepared in phosphate buffer and then diluted at 1 mM with fetal bovine serum (FBS; Biowest, Nuaillé, France). The colloidal suspension was stirred at 37°C for 72 h. Samples withdrawn at different time points (2, 4, 8, 12, 28, 36, 48 and 72 h) were diluted with PBS (1:50 v/v) and analyzed by dynamic light scattering using parameters above reported.

Cell Cultures and viability assays. Two human breast adenocarcinoma cell lines, MDA-MB-231 (ATCC number: HTB26) and MCF7 (ATCC number: HTB22), two human colorectal adenocarcinoma cell lines Caco-2 (ATCC number: HTB-37) and HT29 (ATCC number: HTB-38) and one human prostate cancer PC3 (ATCC number: CLR-1435) have been cultured as previously reported.[3] The human colon cancer Caco-2 and HT29 cell lines were maintained in DMEM medium (Dulbecco's Modified Eagle Medium 1X; GIBCO, Cat No. 31965-023 containing 4.5g/L of D-glucose). The human breast adenocarcinoma cell line MCF-7 (ATCC number: HTB-22) was maintained in DMEM medium (Dulbecco's Modified Eagle Medium 1X; GIBCO, Cat No. 31965-023 containing 1 g/L of D-glucose). The human prostate adenocarcinoma cell line PC3 (ATCC number: CRL-1435) cell line was grown in DMEM/F12 medium (Dulbecco's Modified Eagle Medium Ham's F12 Nutrient Mixture, 1X; GIBCO, Cat. No. 21331-020), 1.5 mM L-glutamine. Each medium was supplemented with 10% Fetal Bovine Serum (FBS, Cat. No. 10270-106, Life Technologies, Monza Italy) and 100 U/ml of Penicillin-Streptomycin (Cat. No 15140-122, Life Technologies, Monza Italy). The human breast adenocarcinoma cell line MDA-MB-231 was grown in DMEM/F12 medium (Dulbecco's Modified Eagle Medium Ham's F12 Nutrient Mixture, 1X; GIBCO, Cat. No. 21331-020), 1.5 mM L-glutamine, MEM Non-Essential

Amino acid solution 1X (SIGMA M7145). Each medium was supplemented with 10% Fetal Bovine Serum (FBS, Cat. No. 10270-106, Life Technologies, Monza Italy) and 100 U/ml of Penicillin-Streptomycin (Cat. No 15140-122, Life Technologies, Monza Italy).The cell cultures were grown in flasks (75 cm^2) and incubated at 37 °C in humidified atmosphere with 5% of CO_2 and 95% of air. The culture medium was changed twice a week. MTT Colorimetric Assay has been performed for viability assays Human cancer cell lines (1.8-3.0 x 10^3 cells/0.33cm^2), were plated in 96 well plates "Nunclon TM Microwell TM" (Nunc), and were incubated at 37 °C. After 24 hours cells (60% confluence) were treated with experimental substances, with or without copper(II) nitrate or zinc chloride, in standard growth medium. Microplates were incubated at 37 °C in humidified atmosphere of 5 % CO_2, 95 % air for 3 days and a colorimetric assay based on the use of tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)[4] was used in order to evaluate the cellular growth inhibition (GI) by the test compound. The results were read on a multiwell scanning spectrophotometer (PlateReader AF2200, Eppendorf, Milan, Italy), using a wavelength of 570 nm. Each value was the average of 4-6 wells. The percentage of cellular growth was calculated according to NCI:[5] $100\times(T - T_0)/(C - T_0)$ (where T is the optical density of the test well after a 72 h period of exposure to test compound, T_0 is the optical density at time zero, C is the optical density of the untreated control cell cultures). When the optical density of treated cells was lower than the T_0 value the following formula was used: $100\times(T-T_0)/T_0$. GI_{50} (50 % growth inhibition) values were calculated by the software GraphPad Prism v.5 with the equation log[inhibitor] *vs.* normalized response.

Electron Spin Resonance spectroscopy (ESR). A Bruker Elexsys E500 CW-EPR spectrometer driven by a PC running the XEpr software and equipped with a Super-X microwave bridge operating at 9.3-9.5 GHz and a SHQE cavity was used throughout this work. Copper(II) complexes were prepared by adding the proper amount of $^{63}Cu(NO_3)_2$ 0.05 M to the micelle/liposome suspension. All ESR spectra of frozen suspension of Cu(II) complexes were recorded at 150 K by means of a ER4131VT variable temperature apparatus. Usually, in order to increase spectral resolution, a little amount of methanol or glycerol (not exceeding 10%) is added to the copper(II) complex, but to prevent any alteration of the

micellar/liposomal structure the suspension was avoided. EPR magnetic parameters calculated from the 2nd and the 3rd line to get rid of second order effects.[6]

Carnosinase hydrolysis resistance assay. The carnosinase activity has been assayed by using an experimental procedure previously reported.[7] Briefly, each sample has been incubated with CN1 5nM at 37°C for 1h. The content of histidine has been measured by means of a spectrofluorimetric assay. Free histidine selectively reacts with o-phthaldialdehyde (OPA) at basic pH and the histidine-OPA adduct, stabilized in acidic conditions, is detected by fluorescence measurements (λ_{exc}: 340 nm, λ_{em}: 450 nm).

RESULTS

Amphiphile derivatives. Amphiphilic derivative of the dipeptide carnosine, $(C18)_2$-L2-Car, was fully synthesized on solid support using the well assessed Fmoc/tBu protocols of the solid phase synthesis. After cleavage from the resin, crude product was precipitated with cold water, purified by RP-HPLC chromatography and characterized by ESI mass spectrometry (Figure S1).

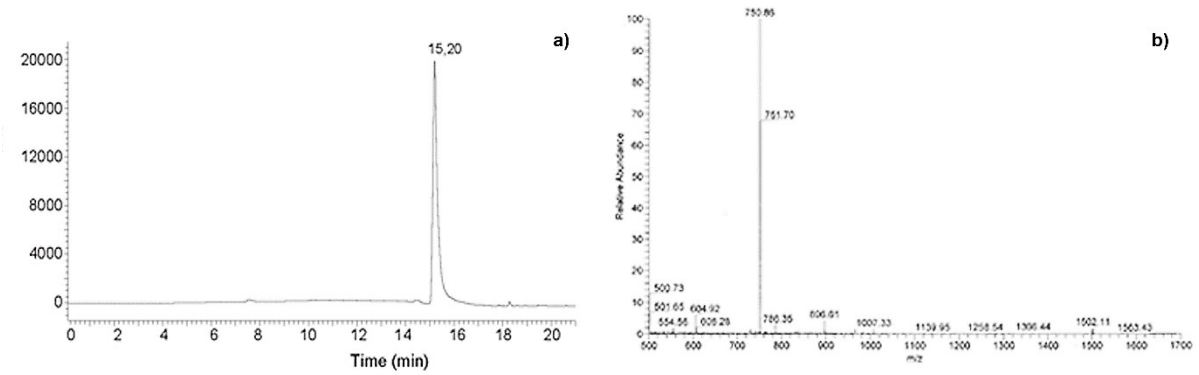

Figure S1. Chromatographic profile of $(C18)_2$-L2-Car amphiphile (a) and its mass spectrum, m/z 750.85 (b).

The insertion of two eighteen carbon atom alkyl chains (C18) at the N-terminus of the peptide sequence permits the anchorage of the carnosine to supramolecular aggregates such as micelles or liposomes. By simply dissolving the $(C18)_2$-L2-Car peptidic amphiphile in aqueous solution a self-assembly of the PA in micelles occurs. As alternative, liposomal aggregates were prepared by adding the commercial

phospholipid (egg PC) to the PA. Similarly to the N,N-dioctadecylsuccinic acid bounded on the N-terminus of the carnosine, also egg PC contains two partially saturated C18 alkyl chains.

This characteristic makes easy the preparation of the supramolecular aggregates (micelles and liposomes) the properties of which were assessed by DLS measurements.

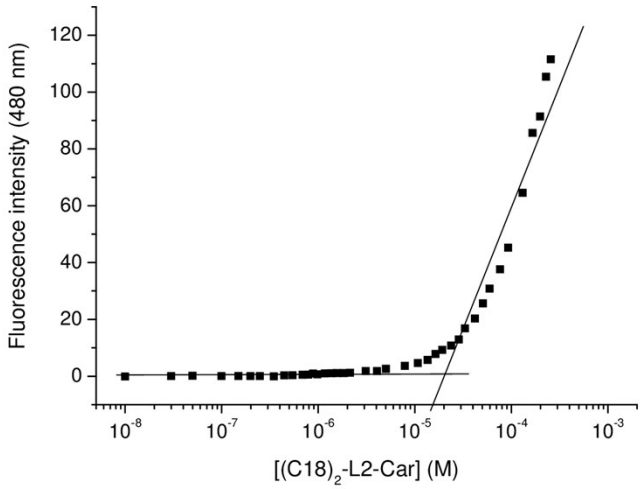

Figure S2. Fluorescence intensity of the ANS fluorophore at 480 nm as a function of $(C18)_2$-L2-Car concentration. CMC value ($2.0 \cdot 10^{-5}$ M) is established from graphical break point.

Moreover, critical micellar concentration (CMC) value for pure micelles $(C18)_2$-L2-Car was calculated using the fluorescence produced by ANS fluorescent probe. The fluorescence intensity of ANS depends on the surrounding environment, being its emission triggered by binding to an hydrophobic environment or substrate whereas water or aqueous solution cause a fluorescence quenching. From the experimental point of view, an ANS solution (2.0×10^{-5} M) in cuvette was titrated with a stock solution of $(C18)_2$-L2-Car and the relative fluorescence intensity at its emission maximum of 480 nm was followed as function of the carnosine derivative concentration. CMC value (2.0×10^{-5} mol kg^{-1}) can be estimated by the graphical break-point, reported in Figure S2.

This value is in good agreement with values previously found for other peptide amphiphiles containing the same hydrophobic.[7 8]

Immunoliposome synthesis

Immunoliposomes were prepared by functionalizing liposomal surface with Anti-HER2-Fab'

fragment using the Micheal addition. Carnosine derivatized liposomes composed of PC/Chol/$(C18)_2$-L2-Car/DSPE-Peg2000-Maleimide at 58.5/35/5/1.5 molar ratio, were prepared in 0.1 M phosphate buffer at 20 mM concentration using the well-known thin lipid film method. Before addition of Fab' fragment to the liposomal solution, 1 equivalent of a reducing agent, TCEP, was incubated with the antibody fragment solution (2.5 mg/mL) for 20 minutes. Derivatization of the liposomal surface, containing maleimido functions was easily achieved overnight and under nitrogen atmosphere. After the reactive immunoliposomes were purified by gel-filtration on CL-4B column, pre-equilibrated with 0.1 M phosphate buffer. The amount of unreacted antibody fragment, evaluated by spectrophotometric analyses, was lower that 5%.

DLS characterization

Intensity profiles and structural data (mean diameter, the diffusion coefficients, *D,* and the polydispersity indexes, PDI) for buffered solutions of micelles, liposomes and immunoliposomes were collected by DLS measurements and summarized in Figure S3, S4 and Table S1, respectively.

Measurements were performed at θ = 173° at a final concentration of $2 \cdot 10^{-4}$ mol·Kg^{-1}. Both kinds of aggregated show a monomodal distribution, which indicates the presence of one population of aggregates. The Stokes-Einstein equation (1) is used to evaluate the hydrodynamic radius, R_H, at infinite dilution

$$R_H = \frac{K_B T}{6\pi\eta D_0} \qquad (1)$$

where D_0 is the translational diffusion coefficient at infinite dilution, K_B is the Boltzmann constant, T is the absolute temperature, and η is the solvent viscosity. Due to the high solution dilution (C = $1 \cdot 10^{-4}$ M) of the systems under study, we have approximately $D \sim D_0$, and equation (1) can be reasonably used to estimate the hydrodynamic radius of the aggregates.

The mean diameter value of ~19 nm, obtained for self-assembles aggregates of $(C18)_2$-L2-Car (see Table S1), are compatible with micellar aggregates, whereas all the others aggregates have a diameter around 100 nm, compatible with liposomal aggregates. From the inspection of the Figure S3, it can be observed

as the insertion of the carnosine derivative in the liposomes (Lip-Car) causes a decrease in the size of liposomes of ~ 30% (Lipo). Instead a slight increase of the size of the liposomes (~ 10%) was observed after the functionalization of the liposomal surface with the Anti-HER2-Fab' fragment (Figure S4). Moreover, any difference in the liposomal size was observed after the coordination of Cu^{2+} or Zn^{2+} metal ions.

Table S1. Structural parameters (mean diameters, polydispersity indexes, PDI, and diffusion coefficients, D) from dynamic light scattering measurements of pure micelles $(C18)_2$-L2-Car, liposomes and immunoliposomes. Measurements were performed on samples at a final concentration of $2 \cdot 10^{-4}$ M.

Systems	Diameter (nm) ± S.D.	PDI	D $[m^2s^{-1}]\times10^{-12}$
$(C18)_2$-L2-Car	19 ± 6.4	0.195	26 ± 8.6
PC/Chol	122 ± 43	0.082	4.0 ± 1.4
PC/Chol/$(C18)_2$-L2-Car	87 ± 42	0.143	5.6 ± 2.7
PC/Chol/$(C18)_2$-L2-Car/DSPE-PEG2000-Mal	95 ± 47	0.150	5.2 ± 2.5
PC/Chol/$(C18)_2$-L2-Car/DSPE-PEG2000-Anti-Her2	104 ± 48	0.153	4.7 ± 2.2

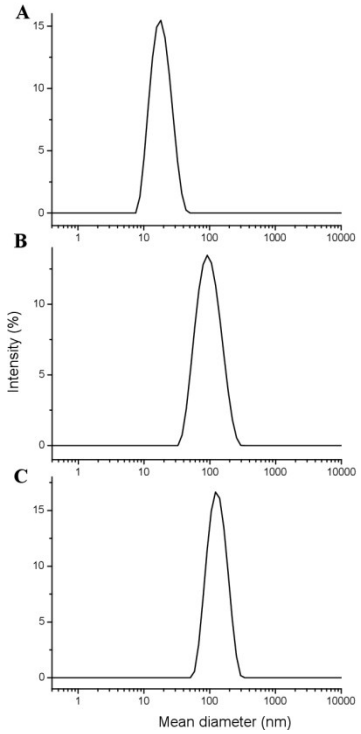

Figure S3. DLS profiles of: A) pure micelles of $(C18)_2$-L2-Car; B) mixed liposomes PC/Chol (60/40) and C) PC/Chol/$(C18)_2$-L2-Car (60/35/5) at the final concentration of $2.0 \cdot 10^{-4}$ M.

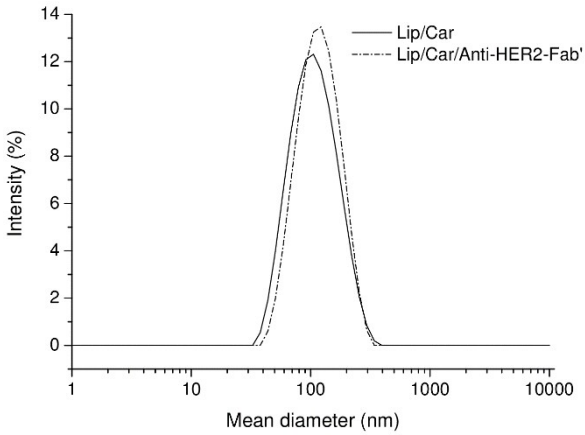

Figure S4. DLS profiles liposomes (Lip/Car and Lip/Car/ Anti-HER2-Fab') before and after functionalization with antibody fragment at the final concentration of $2.0 \cdot 10^{-4}$ M.

Electron Spin Resonance spectroscopy. Liposomes and micelles containing the amphiphilic derivative of the carnosine were studied by ESR in the presence of Cu^{2+} to ascertain if the chelating properties of carnosine are retained or altered. Lip-Car and Mic-Car were allowed to stand in the presence of isotopically pure Cu^{2+} added as $^{63}Cu(NO_3)_2$ at the final concentration of 5×10^{-4} M. To avoid any kind of interference neither methanol nor glycerol was added to the samples to improve the resolution of the ESR spectra. The ESR spectra run on frozen solution of Cu-Lip-Car and Cu-Mic-Car systems were very similar, as can be seen from Figure S5. The second parallel features is more pronounced and could be due to absence of added methanol/glycerol that did not allow the formation of a perfect glass. Nevertheless, the Hamiltonian parameters can be drawn directly from these spectra without any ambiguity. Therefore, carnosine molecules are sufficiently separated on the surface of both micelles and liposomes, so as the Cu^{2+} paramagnetic centres did not interact each other enough to hinder the clear acquisition of the ESR spectra. In particular, both spectra exhibit a $g_{||} = 2.256$ and a $A_{||} = 0.0184$ cm^{-1} which are typical of the monomeric form of copper(II)-carnosine complex.[9] It is known that high carnosine to metal ratios favor the formation of the monomeric copper(II) complex, whereas ratios close to the unity shift the equilibrium towards the formation of the dimeric form.[9,10]

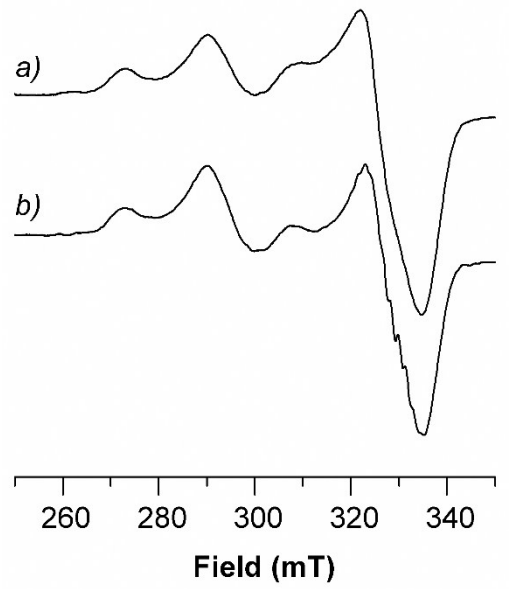

Figure S5. Frozen-solution EPR spectra at 150 K of a buffered (phosphate buffer 0.1 M pH 7.4) aqueous suspension of a) Cu-Mic-CAR system; b) Cu-Lip-CAR system. [Mic-CAR]= 2×10^{-3} M, [Lip-CAR]=0.1 M, $[Cu^{2+}]=5\times10^{-4}$ M

Free carnosine form dimeric species with Cu^{2+} and each metal ion is coordinated by the amino group, an amide nitrogen, a carboxylic oxygen and an imidazole nitrogen coming from the second carnosine molecule (i.e. $Cu_2L_2H_{-2}$ species).[9,10] Interestingly, despite of equimolar addition of Cu^{2+} to both Lip/Car and Mic/Car, only monomeric species were detected by ESR. This finding suggests that on the micelle/liposome surface, carnosine peptide units linked to the alkyl chains are sufficiently far apart from each other to allow each carnosine molecule to coordinate one Cu^{2+} ion alone, being ruled out any mutual interaction.

Carnosinase hydrolysis resistance assay. As reported in the graph below (Figure S6), the histidine content within the carnosine-containing sample (car) gradually forms in 1h due to the carnosinase (CN1) activity. The PEG-linked carnosine, both in micelles and liposomes, is clearly not hydrolyzed by CN1.

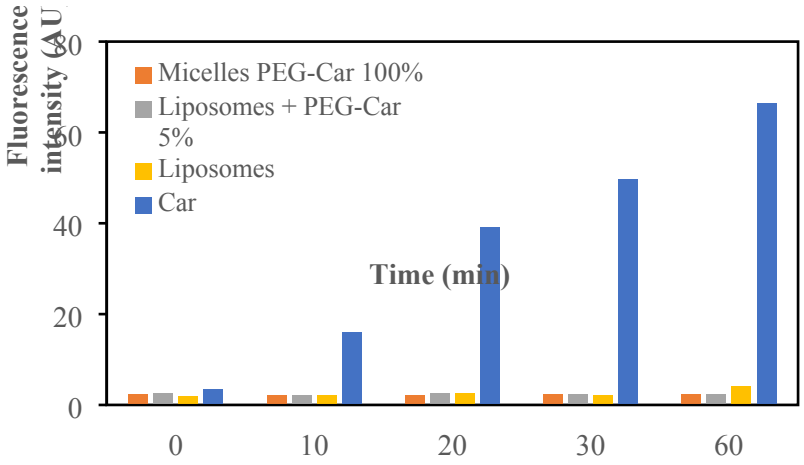

Figure S6. Fluorimetric carnosinase (CN1) activity time course for carnosine PEG-linked either to micelles or liposomes compared to that obtained for free carnosine taken as reference. Non-functionalized liposomes was also taken as additional control.

Biological characterization. Functionalized liposomes were added to the tumor cell (cultured in a multiwell plate) with one-order magnitude step final concentration of 0.005 μM (5 nM), 0.050 μM (50 nM), 0.500 μM, 5 μM and 50 μM. Functionalized liposomes were assayed in the presence and in the absence of Cu^{2+} 10 μM, and in the case of some resistant lines (MCF7 and PC3), also in the presence of Zn^{2+} 50 μM. The cytotoxicity of all drugs was evaluated by the colorimetric assay based on MTT and the results obtained from MDA-MB-231 (HTB26) Adenocarcinoma Mammario, MCF7 (HTB22) Adenocarcinoma Mammario, Caco2 (HTB37) Adenocarcinoma Colon-retto, HT29 (HTB38) Adenocarcinoma del Colon, PC3 (CLR-1435) Adenocarcinoma prostatico. Following are reported the cell growth profiles as a function of the added drug concentration logarithm obtained from all five malignant cell lines.

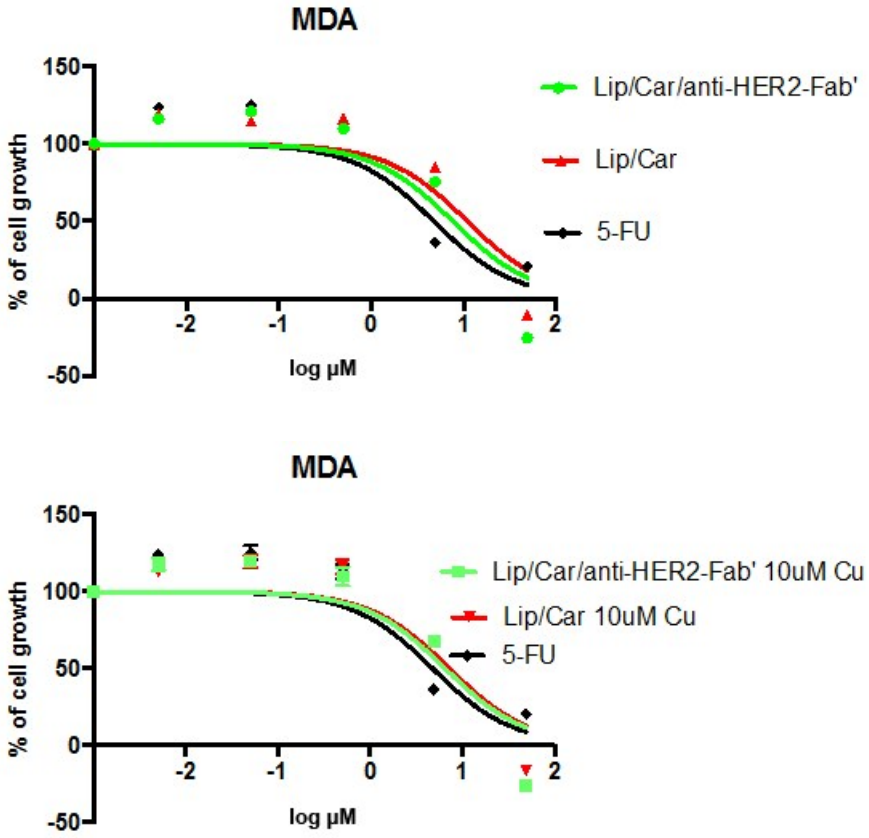

Figure S7. Inhibition profiles of MDA-MB-231 cell culture growth as function of various functionalized liposomes in the presence and in the absence of Cu^{2+} compared to that obtained for 5-FU reference drug.

Table S2. I_{50} values obtained from inhibition profiles of MDA-MB-231 cell culture after addition of functionalized liposomes in the presence and in the absence of Cu^{2+}. 5-FU reference drug value is included for comparison.

	Lip/Car	Lip/Car/Anti-HER2-Fab'	Lip/Car + 10 µM Cu^{2+}	Lip/Car/Anti-HER2-Fab' + 10 µM Cu^{2+}	5-FU
I_{50}	10.94	7.62	7.23	6.51	4.75
95% C. I.	5.09-23.52	3.30-17.64	3.55-14.74	2.92-14.56	2.31-9.77

C.I. – Confidence Interval

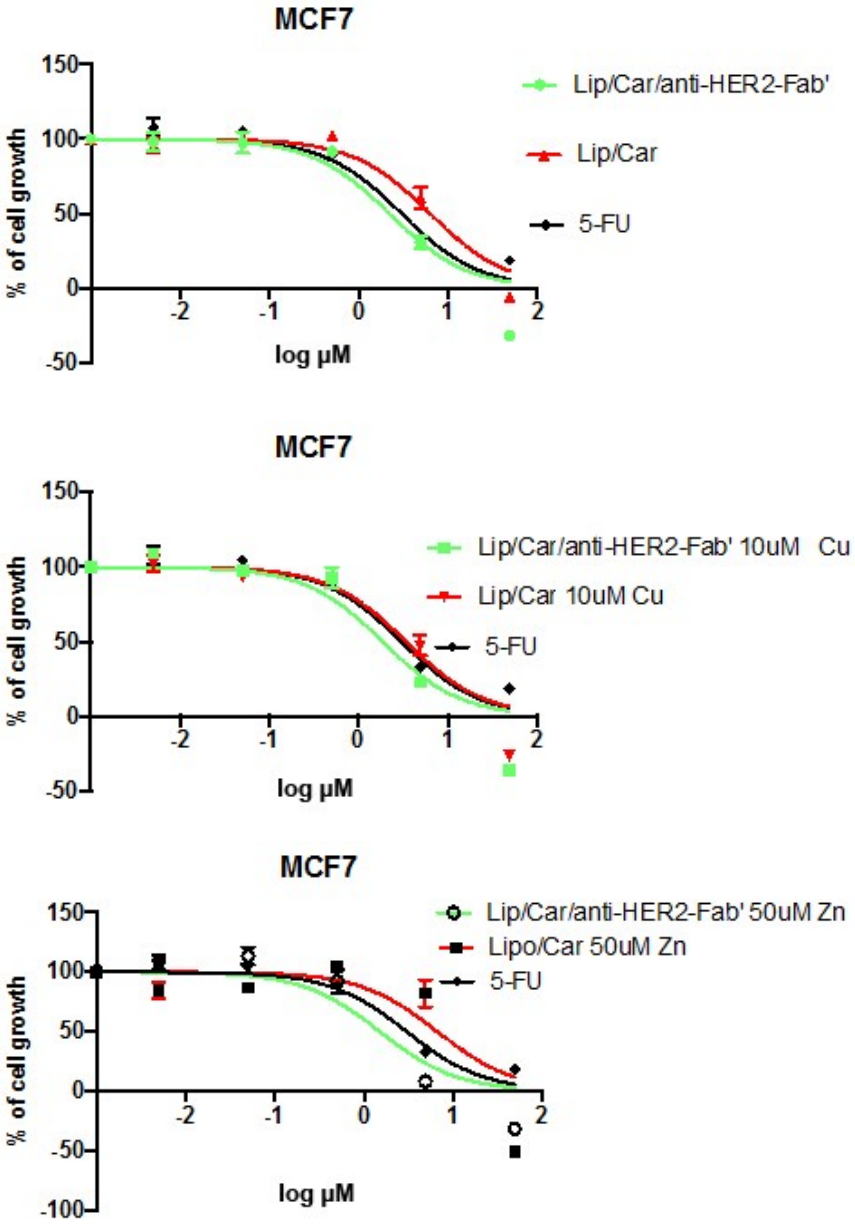

Figure S8. Inhibition profiles of MCF7 cell culture growth as function of various functionalized liposomes in the presence and in the absence of Cu^{2+} or Zn^{2+} compared to that obtained for 5-FU reference drug.

Table S3. I_{50} values obtained from inhibition profiles of MCF7 cell culture after addition of functionalized liposomes in the presence and in the absence of Cu^{2+} or Zn^{2+}. 5-FU reference drug value is included for comparison.

	Lip/Car	Lip/Car/Anti-HER2-Fab'	Lip/Car + 10 µM Cu^{2+}	Lip/Car/Anti-HER2-Fab' + 10 µM Cu^{2+}	5-FU	Lip/Car + 50 µM Zn^{2+}	Lip/Car/Anti-HER2-Fab' + 50 µM Zn^{2+}
I_{50}	6.30	2.19	3.41	1.87	3.02	6.64	1.50
95% C.I.	4.47-8.89	1.17-4.12	1.95-6.00	0.91-3.85	2.24-4.06	2.13-20.73	0.69-3.27

C.I. – Confidence Interval

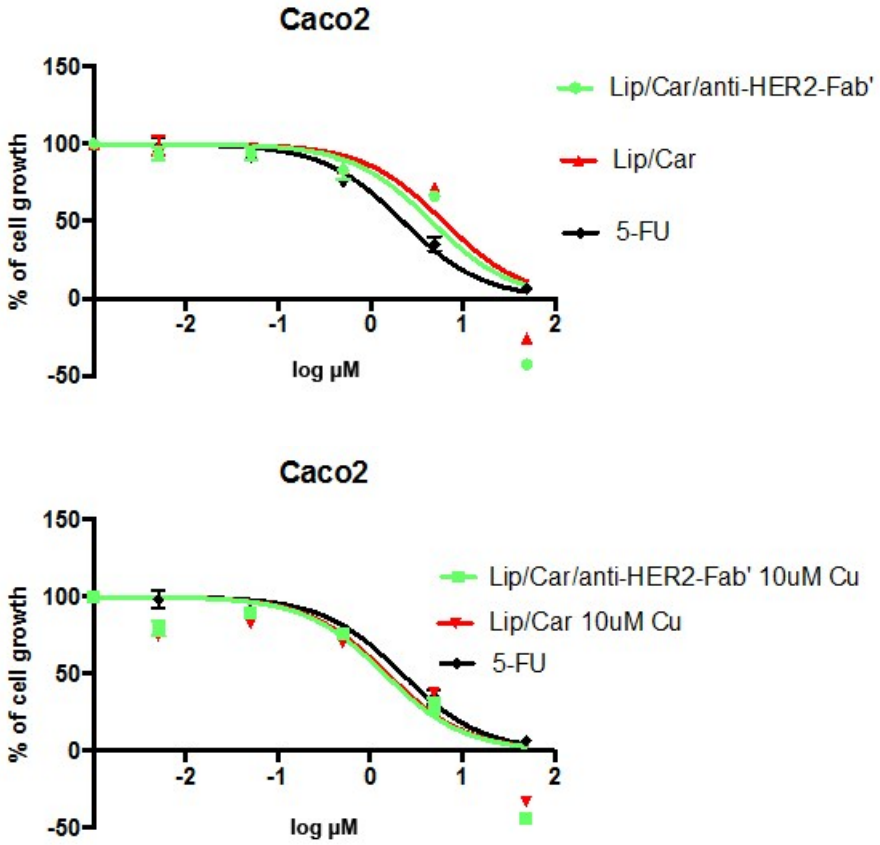

Figure S9. Inhibition profiles of Caco2 cell culture growth as function of various functionalized liposomes in the presence and in the absence of Cu^{2+} compared to that obtained for 5-FU reference drug.

Table S4. I_{50} values obtained from inhibition profiles of Caco2 cell culture after addition of functionalized liposomes in the presence and in the absence of Cu^{2+}. 5-FU reference drug value is included for comparison

	Lip/Car	Lip/Car/Anti-HER2-Fab'	Lip/Car + 10 µM Cu^{2+}	Lip/Car/Anti-HER2-Fab' + 10 µM Cu^{2+}	5-FU
I_{50}	6.08	4.50	1.52	1.42	2.21
95% C. I.	3.15-11.75	1.88-10.77	0.69-3.40	0.60-3.35	1.81-2.71

C.I. – Confidence Interval

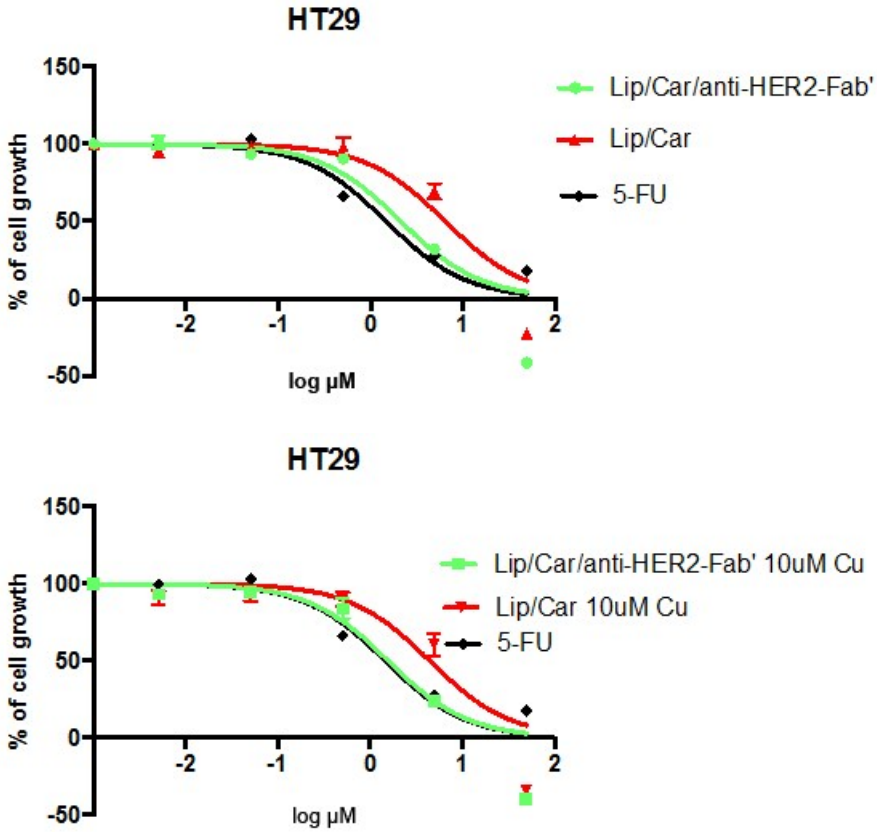

Figure S10. Inhibition profiles of HT29 cell culture growth as function of various functionalized liposomes in the presence and in the absence of Cu^{2+} compared to that obtained for 5-FU reference drug.

Table S5. I_{50} values obtained from inhibition profiles of HT29 cell culture after addition of functionalized liposomes in the presence and in the absence of Cu^{2+}. 5-FU reference drug value is included for comparison

	Lip/Car	Lip/Car/Anti-HER2-Fab'	Lip/Car + 10 µM Cu^{2+}	Lip/Car/Anti-HER2-Fab' + 10 µM Cu^{2+}	5-FU
GI_{50}	6.37	2.10	4.42	1.56	1.46
95% C. I.	3.50-11.61	0.98-4.52	2.14-9.13	0.75-3.26	1.06-1.99

C.I. – Confidence Interval

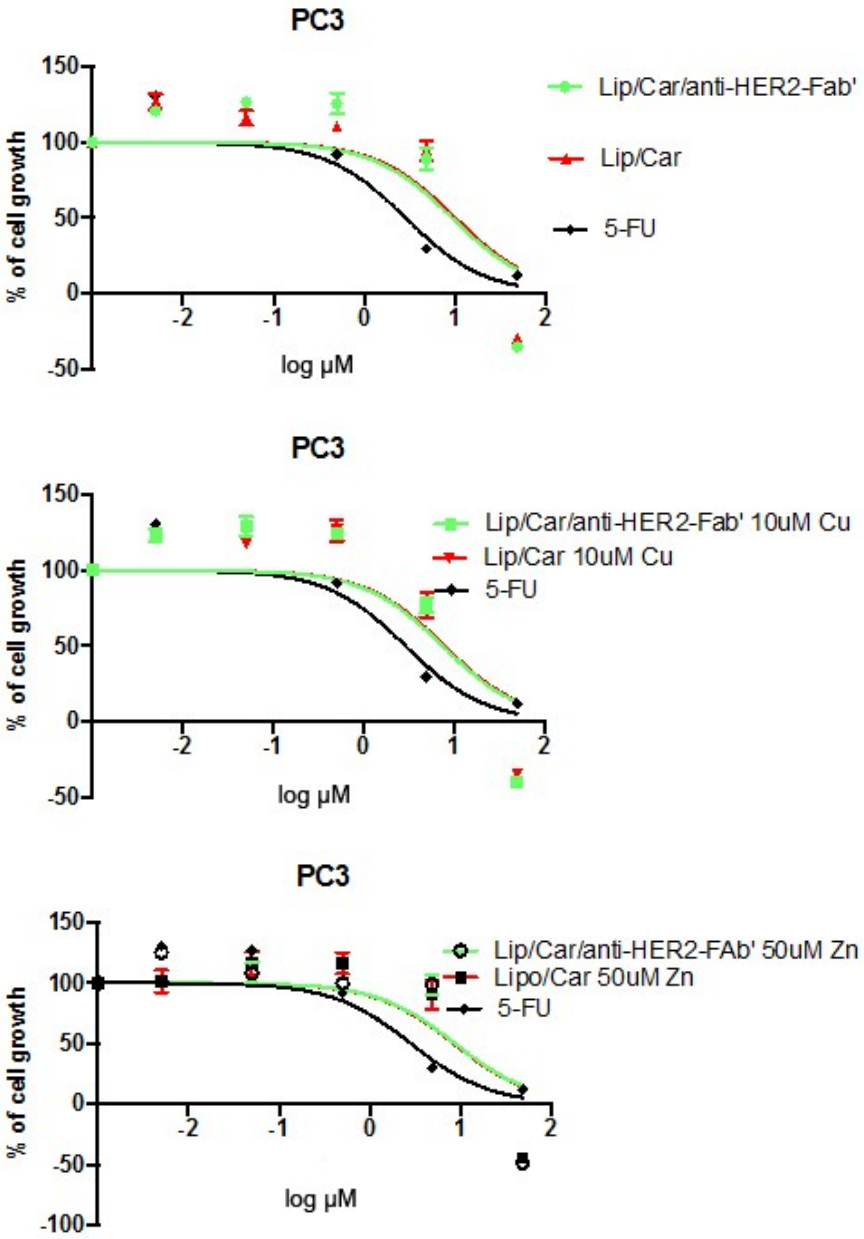

Figure S11. Inhibition profiles of PC3 cell culture growth as function of various functionalized liposomes in the presence and in the absence of Cu^{2+} or Zn^{2+} compared to that obtained for 5-FU reference drug.

Table S6. I_{50} values obtained from inhibition profiles of PC3 cell culture after addition of functionalized liposomes in the presence and in the absence of Cu^{2+} or Zn^{2+}. 5-FU reference drug value is included for comparison.

	Lip/Car	Lip/Car/Anti-HER2-Fab'	Lip/Car + 10 µM Cu^{2+}	Lip/Car/Anti-HER2-Fab' + 10 µM Cu^{2+}	5-FU	Lip/Car + 50 µM Zn^{2+}	Lip/Car/Anti-HER2-Fab' + 50 µM Zn^{2+}
I_{50}	10.16	9.41	7.74	7.38	2.88	8.53	8.78
95% C.I.	3.56-28.99	2.90-30.62	2.64-22.66	2.28-23.87	1.44-5.78	2.65-27.46	2.50-30.83

C.I. – Confidence Interval

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