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Nanoparticles exposing neurotensin tumor-specific drivers‡

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Nanoparticles have attracted much attention for their potential application as *in vivo* carriers of drugs. Labeling of nanoparticles with bioactive markers that are able to direct them toward specific biological target receptors has led to a new generation of drug delivery systems. In particular, low molecular weight peptides that remain stable *in vivo* could be promising tools to selectively drive nanoparticles loaded with active components to tumor cells.

We reported, recently, that tetrabranched neurotensin peptides (NT4) may be used to selectively target tumor cells with liposomes. Liposomes functionalized with tetrabranched neurotensin peptide, NT4, and loaded with doxorubicin showed clear advantages in cell binding, anthracyclin internalization, and cytotoxicity in respect of not functionalized liposomes.

In this study, we compare branched (NT4) *versus* linear (NT) peptides in the ability to drive liposomes to target cells and deliver their toxic cargo. We showed here that the more densely decorated liposomes had a better activity profile in terms of drug delivery. Presentation of peptides to the cell membranes in the grouped shape provided by branched structure facilitates liposome cell binding and fusion. Copyright © 2013 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: tumor-targeting; liposome; branched peptides; doxorubicin

Introduction

Selectivity of therapy is the goal of modern oncology. To achieve tumor selective therapies, identification of tumor 'markers', which should be specific for cancer cells and/or cancer tissue environment, is a fundamental prerequisite together with discovery of selective and effective targeting agents, to be developed as drugs. Antitumor drugs belong to two main categories: (i) those that interfere with a cellular pathway typical of the tumor and (ii) those that bind a tumor-specific molecule and commit the activity to their cytotoxic cargo. Traditional chemotherapeutics such as alkylating agents, antimetabolites, and anthracyclines belong to the former group together with new generation drugs as tyrosine kinase inhibitors [1]. This class also includes antibodies against tumor cell receptors associated to the unregulated growth and division of the tumor cell, such as ErB2 and EGFR [2]. Antitumor drugs of the latter class, in addition to their targeting component, which is typically an antibody or a peptide, also carry a payload like a traditional chemotherapeutic agent [3], or a radioisotope [4], or an immune-stimulating factor [5].

Nanoparticles have attracted much attention for their potential application as *in vivo* carriers of drugs. The use of liposomes as drug carrier systems was proposed by Gregoriadis and Ryman in the early 1970s [6]. These supramolecular aggregates are nontoxic, biodegradable, and nonimmunogenic. Because of their size, which typically ranges in mean diameter of 50–300 nm, liposomes display unique pharmacokinetic properties. These include clearance via the reticuloendothelial system, which results in a relatively long systemic circulation time, as well as hepatic and splenic distribution [7]. Labeling of nanoparticles with bioactive markers that are able to direct them toward specific biological target receptors has led to a new generation of drug delivery systems [8] belonging to the second class of antitumor drugs. In facts, peptides and antibodies are the bioactive markers commonly used to prepare target-selective supramolecular aggregates, such as micelles and liposomes [9–11]. In particular, low molecular weight peptides that remain stable *in vivo* could be promising tools to selectively deliver nanoparticles loaded with active components to tumor cells.

Labeled liposomes loaded with drugs have an advantage *versus* drug-conjugated antibodies or peptides in which the payload they can carry is extremely much larger than that of single molecule. Besides, labeled liposomes allow bypassing the harsh conditions needed for conjugating small molecules to peptides or to antibodies, which may lead to inactivation of the carrier or of the active molecule or both. We reported, recently, that tetrabranched neurotensin peptides (NT4) may be used to selectively target tumor cells with liposomes [12]. Liposomes functionalized with NT4 and loaded with doxorubicin showed clear advantages in cell binding, anthracyclin internalization, and cytotoxicity in respect of not functionalized liposomes. Peptides lie on the liposome surface and need to be properly exposed to be able to bind the target. We wanted to analyze

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the effect of peptide branched structure on target recognition by liposome functionalized with NT4 peptides. It is well known that peptides synthesized in a branched form not only become resistant to proteases but also increase peptide biological activity through multivalent binding [13–17]. In this study, we compare branched (NT4) *versus* linear (NT) peptides in the ability to drive liposomes to target cells and deliver their toxic cargo.

Results and Discussion

Peptide Selection and Liposome Formulation

Tetrabranched neurotensin peptides, NT4, can efficiently discriminate between tumor and healthy tissues in human surgical samples from colon and pancreas adenocarcinoma in a high number of patients, with good statistical significance, whereas monomeric neurotensin peptides, NT, cannot [18,19]. Accordingly, tumor cell binding was extremely much lower for the monomeric NT compared with NT4. Moreover, liposomes filled with doxorubicin (Doxo) resulted much more efficient in intracellular drug delivery when decorated with tetrabranched NT short functional 8–13 fragment compared with analogous undecorated particles.

We want now to verify if facilitated target recognition occurs even when using monomeric peptides or it happens only when neurotensin peptides are in a grouped shape on the external liposome surface. For this purpose, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) liposomes decorated with different amounts of neurotensin monomeric peptides have been prepared by following the procedure already described [12]. The monomeric linear peptides corresponding to the neurotensin entire sequence (NT_{1-13}) and corresponding to the short active sequence (NT_{8-13}) were functionalized at the C-terminus with two ethoxilic linkers (L) and with an hydrophobic moiety, $(C18)_2$, containing two 18-carbon alkyl chains atoms, in order to insert them in DOPC liposomes. The chemical structures of the neurotensin functionalized peptides (NT_{1-13})L-$(C18)_2$ and (NT_{8-13})L-$(C18)_2$ are reported in Figure 1 along with the tetrabranched neurotensin derivative (NT_{8-13})4-L-$(C18)_2$ used in the previous study. Peptide derivatives have been synthesized by solid phase methods using a Rink amide resin [20]. An orthogonal protected lysine residue was bound to the solid support, and then, two Fmoc-21-amino-4,7,10,13,16,19-hexaoxaheneicosanoic acid (Fmoc-Ahoh-OH) spacers and the hydrophobic moiety *N*,*N*-dioctadecylsuccinamic acid were sequentially bound to lysine side chain, whereas the amino acidic sequences of NT_{8-13} or NT_{1-13} were added on the lysine N-α function. Both peptide derivatives (NT_{1-13})L-$(C18)_2$ and (NT_{8-13})L-$(C18)_2$ were purified by HPLC on C4 column and obtained in good yield and high purity.

Pure DOPC liposomes and mixed DOPC/(NT_{1-13})L-$(C18)_2$ and DOPC/(NT_{8-13})L-$(C18)_2$ liposomes at several molar ratios (95:5, 90:10, and 85:15) of the two amphiphilic components were prepared as already reported for DOPC liposomes containing the tetrabranched neurotensin derivative (NT_{8-13})4-L-$(C18)_2$, prepared at 95:5 ratio. The ratio of DOPC and amphiphilic neurotensin derivatives has been modulated to verify if the amount of the exposed peptide could influence cell binding and cytotoxicity of the entire liposomes filled with doxorubicin. In the case of the already studied DOPC/(NT_{8-13})4-L-$(C18)_2$ 95:5 liposomes, four copies of NT_{8-13} peptide are present for each monomer thus corresponding to 80:20 in DOPC/peptide ratio. Preparation of liposomes containing 20% weight of monomeric neurotensin derivatives (80:20 ratio) is not feasible using

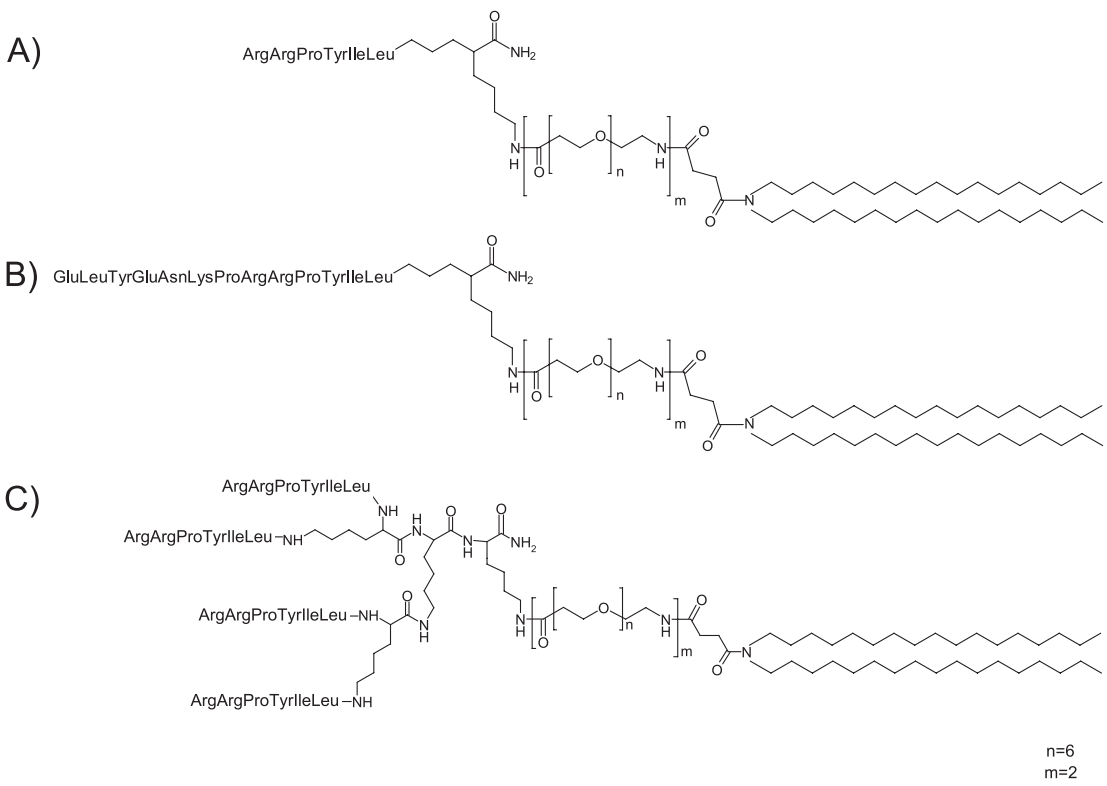

Figure 1. Schematic representation of the neurotensin functionalized peptides (NT_{8-13})L-$(C18)_2$ and (NT_{1-13})L-$(C18)_2$ and of the tetrabranched neurotensin derivative (NT_{8-13})4-L-$(C18)_2$ used in the previous study [12].

monomeric derivatives. Therefore, the total amount of exposed peptides remains, for all the three obtained compositions, lower than in the case of liposomes with tetrabranched derivative.

The size of pure DOPC and mixed DOPC/NT-peptide liposomes at several molar ratios (R = 0, R = 95/5, R = 90/10, and R = 85/15) was assessed by dynamic light scattering (DLS). Measurements were performed at $\theta = 173°$ on liposomes at $5 \cdot 10^{-4}$ M concentration in 0.1 M phosphate buffer at pH 7.4. Hydrodynamic radius (R_H) distribution function of DOPC/(NT_{8-13})L-$(C18)_2$ liposomes are reported in Figure 2, and R_H values and polydispersity index of all systems are summarized in Table 1. All aggregates with the exception of the sample with R = 85/15 showed a distribution clearly mono-modal, i.e. there is a presence of a single mode with a radius of ~ 60 nm (Table 1). These results indicate that the size of DOPC liposomes does not change when low amounts of NT_{8-13} or NT_{1-13}-peptide monomers (≤10%) are added to the formulation. Instead, liposomes containing high percentage of NT_{8-13} or NT_{1-13}-peptide monomers (R = 85/15) present a bimodal distribution with a slow and a fast mode, whose mean radius are 71 ± 21 nm and 28 ± 5 nm, respectively.

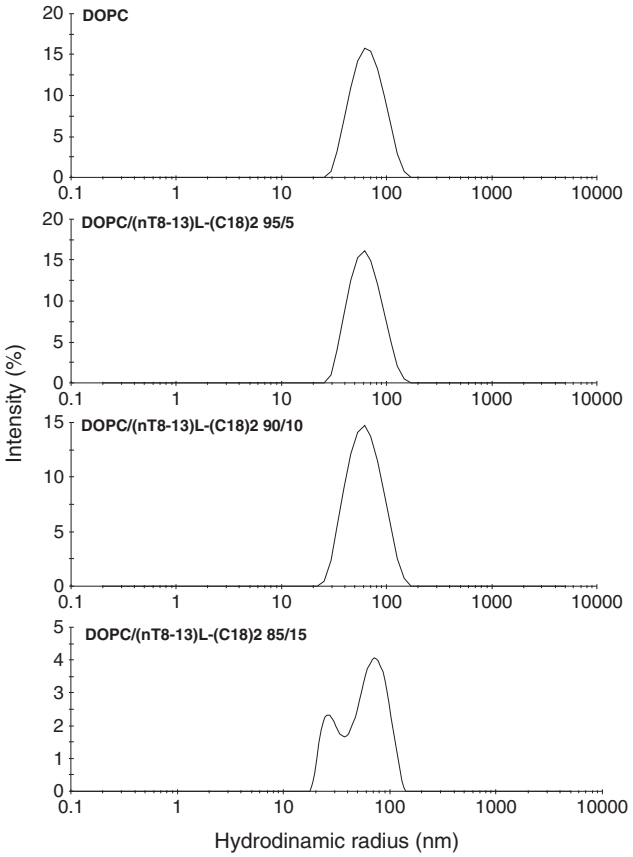

Figure 2. DLS spectra of DOPC/(NT_{8-13})L-(C18)2 systems at different molar ratios (R = 0, 95:5, 90:10, 85:15).

Doxorubicin, which has a dual function being a cytotoxic antibiotic and a fluorescent probe, was loaded into liposomes by using the pH gradient method. The liposomal formulations designated as DOPC/Doxo, DOPC/(NT_{1-13})L-$(C18)_2$/Doxo and DOPC/(NT_{8-13})L-$(C18)_2$/Doxo were obtained and characterized measuring the Doxo loading content (DLC, defined as the weight ratio of encapsulated Doxo *versus* the amphiphilic moieties). The DLC values obtained for mixed liposomes (R = 95/5 and R = 90/10) are comparable with the value found for pure DOPC liposomes, whereas the DLC for liposomes containing 15% of peptide monomer decrease of about 30%. This result clearly indicates that at this molar ratio, several changes occur in liposomal structure (see DLS results), and as a consequence, the amount of encapsulated drug decrease. Because of the low DLC value and to the simultaneous presence of supramolecular structures with different size and shape, mixed DOPC liposomes containing 15% of peptide monomer were kept away from *in vitro* cellular investigations.

Cell Binding and Internalization

Liposomes carrying NT_{1-13} (DOPC/(NT_{1-13})L-$(C18)_2$/Doxo) or NT_{8-13} (DOPC/(NT_{8-13})L-$(C18)_2$/Doxo), at 95:5 and 90:10 molar ratio between the two amphiphilic components, were compared with the analogous liposomes decorated with NT4, DOPC/(NT_{8-13})4-L-$(C18)_2$/Doxo, by confocal microscopy experiments. HT29 and TE671 cells were incubated for 30 min at 25 °C with liposome formulations 1 μM (in the case of liposomes decorated with branched NT) or 5 μM (for liposomes decorated with monomeric NT or for not decorated liposomes) calculated as molarity of Doxo. Figure 3 shows confocal microscopy images and 3D cell surface plot of HT29 and TE671 cells incubated with liposomes decorated either with monomeric NT_{8-13} or with tetrabranched NT_{8-13} or with nude liposomes. Internalized Doxo is much higher when the carrier is a tetrabranched peptide compared with a monomeric peptide. The amount of Doxo brought in by the monomeric-decorated liposomes increases with the increase of peptide

Table 1. Composition of the lipid bilayer and structural parameters (hydrodynamic radii, R_H, and polydispersity index, PI) data of the obtained liposomes

Formulation	Composition of the lipid bilayer	R_H (nm) ± SD	PI ± SD
DOPC/Doxo		61 ± 20	0.079 ± 0.02
DOPC/(NT_{1-13})L-$(C18)_2$/Doxo	95/5	60 ± 23	0.098 ± 0.03
DOPC/(NT_{1-13})L-$(C18)_2$/Doxo	90/10	58 ± 21	0.101 ± 0.03
DOPC/(NT_{1-13})L-$(C18)_2$/Doxo	85/15	89 ± 27 23 ± 4	0.244 ± 0.05
DOPC/(NT_{8-13})L-$(C18)_2$/Doxo	95/5	59 ± 27	0.122 ± 0.03
DOPC/(NT_{8-13})L-$(C18)_2$/Doxo	90/10	57 ± 24	0.110 ± 0.03
DOPC/(NT_{8-13})L-$(C18)_2$/Doxo	85/15	71 ± 21 28 ± 5	0.194 ± 0.05

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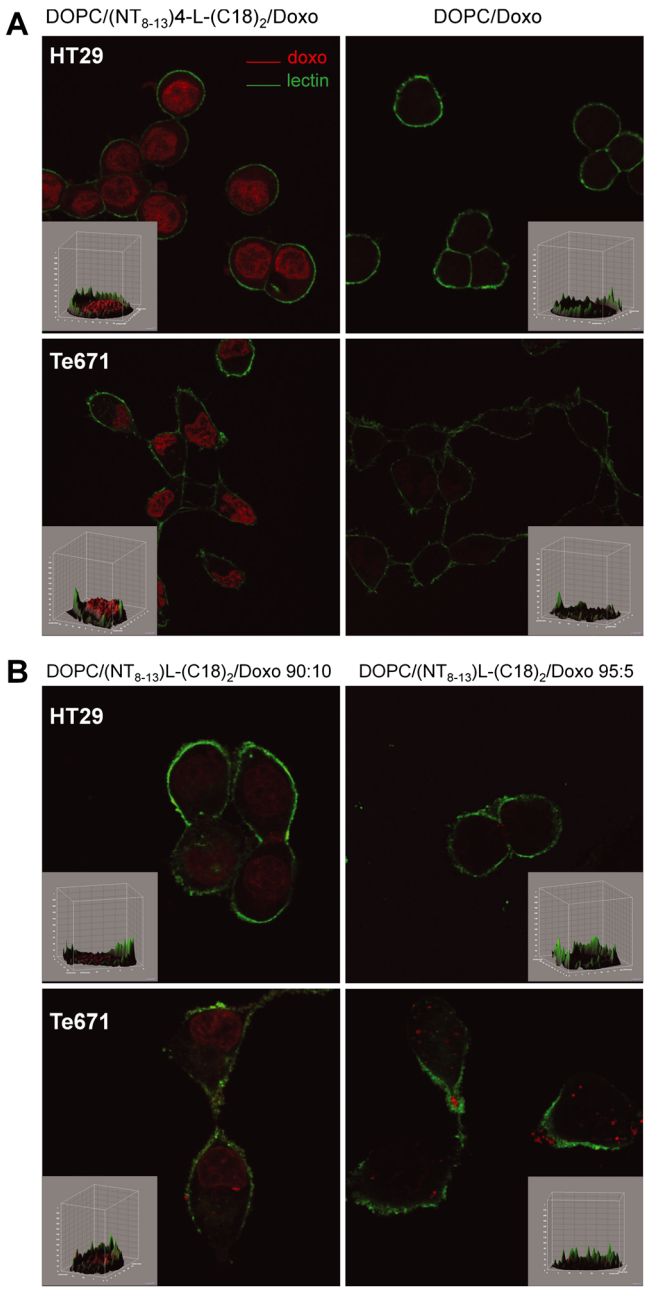

Figure 3. Liposomes binding on HT29 and TE671 cell lines. HT29 and TE671 incubated with DOPC/(NT_{8-13})4-L-$(C18)_2$/Doxo, DOPC/Doxo (panel A) or with DOPC/(NT_{8-13})L-$(C18)_2$/Doxo 90:10, DOPC/(NT_{8-13})L-$(C18)_2$/Doxo 95:5 (panel B) analyzed with confocal microscopy. Cell membrane is stained with Lectin-Atto 647.

on the liposome surface being equal to that of nondecorated particles of 95/5 molar ratio (Figure 3)

Cytotoxicity

HT29 and TE671 cells were incubated with various concentrations, from 8 nM to 25 μM, of DOPC/(NT_{1-13})L-$(C18)_2$/Doxo 90:10, DOPC/(NT_{8-13})L-$(C18)_2$/Doxo 90:10, or DOPC/(NT_{8-13})4-L-$(C18)_2$/Doxo. After 8 h of incubation, cells were washed and incubated for 3 days. Washing was performed to avoid diffusion of free Doxo from the liposomes during the 3-day incubation period. As reported in Figure 4, DOPC/(NT_{1-13})L-$(C18)_2$/Doxo and DOPC/(NT_{8-13})L-$(C18)_2$/Doxo exert a very mild cytotoxic effect when compared with the branched-decorated DOPC/(NT_{8-13})4-L-$(C18)_2$/Doxo liposomes (Figure 4) [11]. In facts, in HT29 *in vitro* model, there is more than one log of difference between DOPC/(NT_{8-13})4-L-$(C18)_2$/Doxo and DOPC/(NT_{1-13})L-$(C18)_2$/Doxo or DOPC/(NT_{8-13})L-$(C18)_2$/Doxo. In TE671, again, DOPC/(NT_{8-13})4-L-$(C18)_2$/Doxo is at least eight times better than DOPC/(NT_{1-13})L-$(C18)_2$/Doxo or DOPC/(NT_{8-13})L-$(C18)_2$/Doxo.

1,2-Dioleoyl-sn-glycero-3-phosphocholine mixed liposomes containing 5% of peptide monomer, DOPC/(NT_{1-13})L-$(C18)_2$/Doxo 95:5 and DOPC/(NT_{8-13})L-$(C18)_2$/Doxo 95:5, were also examined; results indicate that in this experimental conditions, where long-time incubation may have a leveling effect, no differences were detectable when compared with the 90:10 analogs reported previously.

Flow Cytometry

HT29 and TE671 cells were incubated with 5 μM liposomes decorated with monomeric NT or with 1 μM liposomes decorated with branched NT (data not shown). Again, liposomes decorated with monomeric peptides showed much lower efficiency in Doxo delivery to cancer cells compared with those containing branched peptides. To verify whether the ability of liposomes to fuse with the cells and deliver Doxo was related to the surface concentration of peptides, we compared liposomes prepared with different DOPC/peptide monomer ratio. The more densely decorated liposomes DOPC/(NT_{8-13})L-$(C18)_2$/Doxo 90:10 (shown in Figure 5) had a better activity profile than those exposing a lower concentration of peptide (DOPC/(NT_{8-13})L-$(C18)_2$/Doxo 95:5) (Figure 5). Identical result was given by DOPC/(NT_{1-13})L-$(C18)_2$/Doxo at two different molar ratios (not shown). The best activity response was again given by branched-decorated liposomes, which indeed are those with the highest concentration of peptide on the particle surface.

Material and Methods

Protected N^{α}-Fmoc-amino acid derivatives, coupling reagents, and Rink amide MBHA (methylbenzhydrylamine) resin were purchased from Calbiochem-Novabiochem (Läufelfingen, Switzerland). The Fmoc-21-amino-4,7,10,13,16,19-hexaoxaheneicosanoic acid (Fmoc-Ahoh-OH) was purchased from Neosystem (Strasbourg, France). The *N*,*N*-dioctadecylsuccinamic acid was synthesized according to published methods [21]. (8S,10S)-10-(4-amino-5-hydroxy-6-methyl-tetrahydro-2H-pyran-2-yloxy)-6,8,11-trihydroxy-8-(2-hydroxyacetyl)-1-methoxy-7,8,9,10- tetra hydro-tetracene-5,12-dione; doxorubicin HCl was purchased from Sigma-Aldrich. DOPC was purchased from Avanti Polar Lipids (Alabaster, AL, USA). All other chemicals were commercially available by Sigma-Aldrich (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. Solid phase peptide synthesis was performed on a 433A Applied Biosystems automatic synthesizer (Carlsbad, California). Analytical LC-MS analyses were performed by using Finnigan Surveyor MSQ single quadrupole electrospray ionization (Finnigan/Thermo Electron Corporation San Jose, CA), column: C4-Phenomenex eluted with H_2O/0.1% TFA (A) and

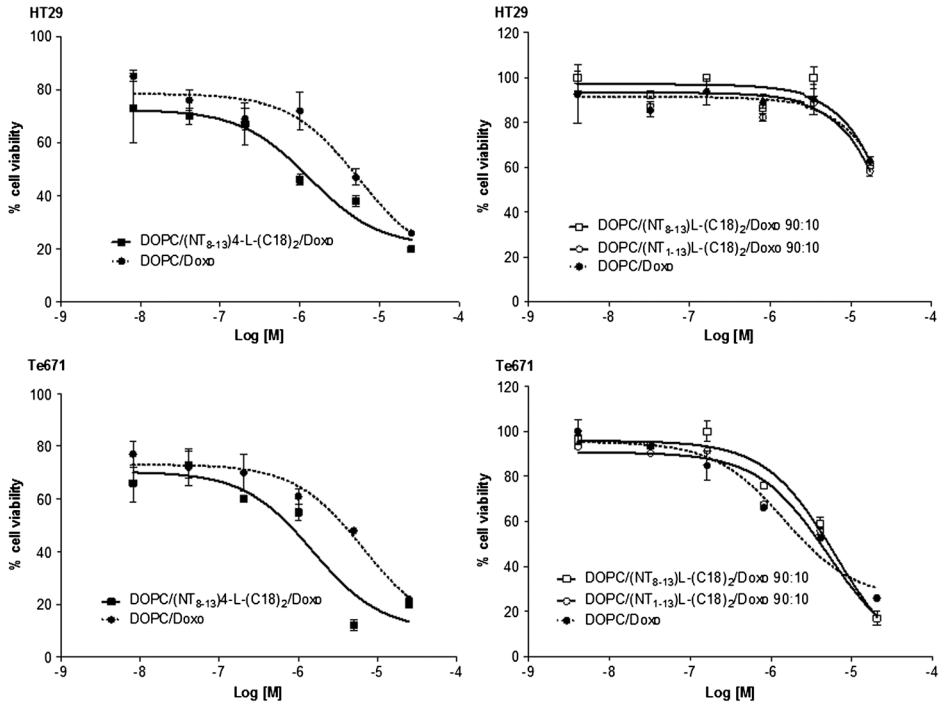

Figure 4. Cytotoxicity HT29 and TE671 cells were incubated with different concentration of liposomes decorated either with tetrabranched NT (left) or with monomeric NT (right) or with nude liposomes (DOPC-doxo). After 8 h of incubation, cells were washed and grown for 3 days.

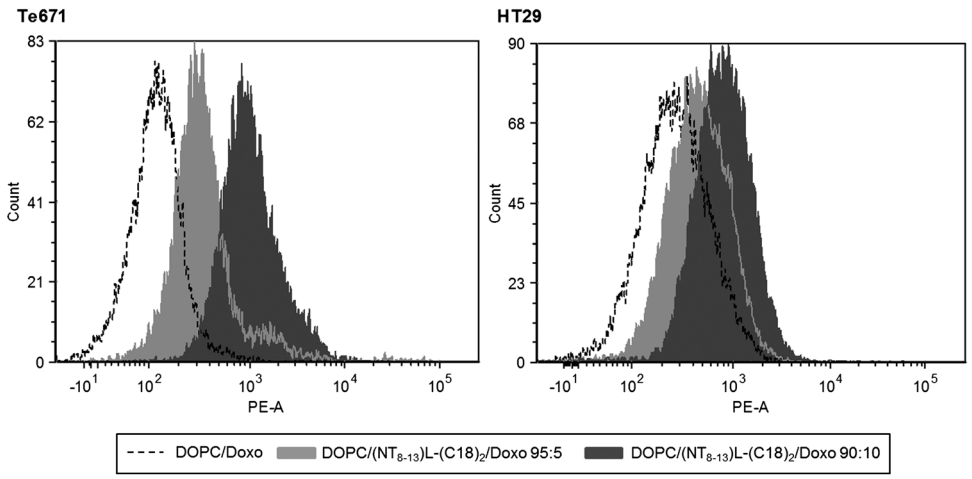

Figure 5. Flow cytometry analysis. HT29 and TE671 cells were incubated with DOPC/(NT_{8-13})L-$(C18)_2$/Doxo 90:10 (black dark gray), DOPC/(NT_{8-13}) L-$(C18)_2$/Doxo 95:5 (light gray) and with DOPC/Doxo liposomes (dotted line). Fluorescent signals were measured with BD FACSCalibur.

CH_3CN/0.1% TFA (B) from 20–80% over 20 min at a flow rate of 0.8 ml/min. The crude peptides were purified by RP-HPLC on an LC8 Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a UV lambda-Max Model 481 detector using a Phenomenex (Torrance, CA) C4 (300 Å, 250 × 21.20 mm, 5 μ) column eluted with H_2O/0.1% TFA (A) and CH3CN/0.1% TFA (B) from 20–80% over 20 min at a flow rate of 20 ml min^{-1}. UV measurements were performed on a UV–vis Jasco V-5505 spectrophotometer (Easton, MD) equipped with a Jasco ETC.-505T Peltier temperature controller with a 1-cm quartz cuvette (Hellma). The liposomes were extruded using a mini-extruder purchased from Avanti Polar Lipids.

Peptide Conjugate Synthesis

Peptide synthesis was carried out in solid phase under standard conditions using Fmoc strategy on Rink amide MBHA resin (0.65 mmol g^{-1}, 0.10 mmol scale, and 0.154 g) [20]. After the Fmoc removal from the resin, the coupling of the Dde-Lys (Fmoc)-OH residue was performed by using HOBt/PyBop/DIPEA

(1/1/2) according to the standard protocols of solid phase peptide synthesis (single coupling with four equivalents of amino acid in DMF for a reaction time of 1 h). Successively, side chain of lysine residue was deprotected with DMF/piperidine mixture (70/30), and the two Fmoc-Ahoh-OH hexaoxoethylene linker and the *N*,*N*-dioctadecylsuccinamic acid were sequentially condensed as previously reported [20]. The lipophilic derivative was coupled after dissolution in DMF/CH_2Cl_2 (50:50). Then, Dde protecting group of the N-terminus of the lysine was removed with DMF/Hydrazine (98/2), and the peptide chain of NT_{8-13} or NT_{1-13} was elongated by sequential coupling by the standard HOBt/PyBop/DIEA procedure with DMF as solvent and Fmoc deprotection of the Fmoc-amino acid derivatives. Peptides were fully deprotected and cleaved from the resin with TFA with 2.5% (v/v) water, 2.5% (v/v) TIS as scavengers, at room temperature, and then precipitated with ice-cold water, filtered, dissolved in water, and lyophilized. The crude peptide derivatives were purified by RP-HPLC. Purity and identity were assessed by analytical LC-MS analyses. The final yields of purified peptides ranged between 20% and 40%.

$(NT_{1-13})L-(C18)_2$ Rt = 16.76 min; MS (ESI) : $[M+2H^+]/2$ = 1546.7 amu; $[M+3H^+]/3$ = 1031.6 amu; $[M+4H^+]/4$ = 773.9 amu (MW : 3091.4).

$(NT_{8-13})L-(C18)_2$ Rt = 12.05 min; MS (ESI) : $[M+2H^+]/2$ = 1105.5 amu; (MW : 2210.2).

Liposome Preparation and DLS Characterization

All solutions were prepared by weight and buffered at pH 7.4 using 0.1 M phosphate buffer. The pH was controlled using pH meter MeterLab PHM220. Mixed aggregates of DOPC/(NT_{1-13})L-$(C18)_2$ and DOPC/(NT_{8-13})L-$(C18)_2$ at several molar ratio (95:5, 90:10, and 85:15) were prepared as reported [12]: the two amphiphiles were dissolved in a small amount of MeOH/$CHCl_3$ (50:50); subsequently, a thin film of amphiphiles was obtained by evaporating the solvent by slowly rotating the tube containing the solution under a stream of nitrogen. Lipid film wasc hydrated by addition of 0.1 M phosphate buffer (pH 7.4), sonicated for 30 min and extruded 21 times through a polycarbonate membrane (100 nm pore size). The same procedure was used also to prepare pure DOPC liposomes. The effective amount of NT_{1-13} or NT_{8-13} peptide enclosed in the several liposomal formulations was confirmed by UV–vis at 270 nm (Tyr residue absorbance). After dilution of samples at $2.0 \cdot 10^{-4}$ M concentration and centrifuged at room temperature at 13 000 rpm for 5 min, hydrodynamic radii (R_H) were measured by DLS. The setup for the DLS measurement was a Zetasizer Nano ZS (Malvern Instruments, Westborough, MA) that employs a 173° backscatter detector. Other instrumental settings are measurement position (mm): 4.65; attenuator: 8; temperature 25 °C; cell: disposable sizing cuvette.

Doxorubicin Loading

1,2-Dioleoyl-sn-glycero-3-phosphocholine/Doxo, DOPC/(NT_{1-13})L-$(C18)_2$/Doxo and DOPC/(NT_{8-13})L-$(C18)_2$/Doxo liposomal formulations were prepared by loading doxorubicin · HCl into liposomes by using the pH gradient method. Briefly, the liposomal solution was prepared as reported previously at pH 4.0 using 0.1 M citrate–phosphate buffer. The pH was adjusted from 4.0 to 7.4 by dropwise addition of a 1.0 M stock solution of NaOH. Next, Doxo was added to liposomal solution from aqueous stock solution in order to have a drug/lipid ratio of 0.1. The suspension was stirred for 30 min at room temperature. The Doxo concentration in all experiments was determined by spectroscopic measurements (UV or fluorescence) using calibration curves obtained by measuring absorbance at 480 nm. Subsequently, unloaded Doxo was removed by gel filtration with a Sephadex G50 column pre-equilibrated with 2.5 mM phosphate buffer at pH 7.4. The drug loading content (DLC, defined as the weight ratio of encapsulated Doxo *versus* the amphiphilic moieties) was quantified by subtraction of the amount of Doxo removed from the total amount of Doxo loaded.

Cell Cultures

HT29 human colon adenocarcinoma and TE671 human rhabdomyosarcoma cells were grown in their recommended medium, respectively, McCoy's 5A or DMEM supplemented with 10% fetal calf serum, 200 μg/ml glutamine, 100 μg/ml streptomycin, 60 μg/ml penicillin, and maintained at 37 °C, 5% CO_2. Cell lines were purchased from Istituto Zooprofilattico Sperimentale (Brescia, Italy).

Cell Binding and Internalization

HT29 and TE671 were plated at the density of 25 000 cells/well and, after overnight adhesion, incubated for 30 min at 25 °C with liposomes diluted in completed medium. Liposomes concentration, calculated as molarity of Doxo, was 1 μM for liposomes decorated with branched NT, 5 μM for liposomes decorated with monomeric NT or for not decorated liposomes. Cells were fixed for 10 min in 4% PFA-TBS (Tris 50 mM NaCl 150 mM pH 7.4), and then, the cellular membrane was stained with lectin-Atto 647 0.5 μg/ml in 0.3% BSA–TBS for 15 min at 25 °C. Each step was followed by three washes with TBS. Images were taken with a TCS SP5 Leica confocal microscope (514λ_{ex} 550–600 λ_{em} for doxo; 633 λ_{ex} 660–690 λ_{em} for Atto 647). All images were then analyzed with ImageJ software. Experiments were repeated three times, at least.

Cytotoxicity

HT29 and TE671 were plated at a density of 5000 cells/well in 96-well microplates and grown for 24 h and then were exposed to different concentration of liposomes (8 nM–25 μM). After 8 h of incubation, cells were washed and grown for 3 days at 37 °C with complete medium. 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), 50 μg/ml was then added for 3.5 h at 37 °C. Cells are then lysed with DMSO, and the absorbance of MTT salts of living cells was read at a spectrophotometer. Results were analyzed by nonlinear regression

analysis using GraphPaD Prism 5.03 software. Experiments were repeated three times, at least.

Flow Cytometry Analysis

The various liposomes preparations with different ratio of DOPC/peptide monomer were incubated with HT29 and TE671 cells and analyzed by flow cytometry. Briefly, 100 000 cells/well were incubated in 96 well U-bottom plates for 30 min at 25 °C with 5 μM of DOPC/(NT_{8-13})4 L-$(C18)_2$/Doxo, DOPC/(NT_{8-13}) L-$(C18)_2$/Doxo, and DOPC/(NT_{1-13})L-$(C18)_2$/Doxo at the molar ratios of 95:5, 90:10, and DOPC/Doxo, diluted in completed medium and then fixed for 10 min with 4% PFA-TBS. Flow cytometry measurements were obtained analyzing 20 000 events with a BD FACSCalibur (BD, NJ. USA). Experiments were repeated three times, at least.

Conclusions

Labeled nanoparticles are of growing interest in the field of drug delivery system. These are very smart new drugs that can be directed against selective targets. In this study, we demonstrated that it is not only important to choose the right bioactive marker that brings the molecule to its target but also the mode of exposure of the carrier on the particle surface, and its amount, may be crucial.

We showed here that the more densely decorated liposomes had a better activity profile in terms of drug delivery. Unfortunately, preparation of liposomes containing 15% or 20% weight of monomeric neurotensin derivatives could not be obtained by using monomeric peptide derivatives. We compared growing concentrations of linear peptides and observed that the increase in peptide exposure correlated with an increased activity. Nonetheless, a high concentration of liposome-inserted peptides resulted in a drop of liposome stability.

On the other hand, the use of branched peptides in the amphiphilic monomer yields a higher concentration of peptide, i.e. a lower DOPC/peptide ratio. As a result, liposome decorated with branched peptides has a better profile in drug delivery, with respect to liposomes decorated with correspondent monomeric peptides. This achievement might be seen as a general approach to increase the exposure of carriers on the surface of the nanoparticles, which is a frequently wanted matter. The use of multimeric peptides as decorating units of nanoparticle has, eventually, the merit of increasing the amount of hydrophilic carrier to be exposed to the target cell. Besides, the presentation of peptides in a grouped shape facilitates binding and fusion of particles to cells.

Acknowledgements

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