DOI: 10.1002/cmdc.201000463

Target-Selective Drug Delivery through Liposomes Labeled with Oligobranched Neurotensin Peptides

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The structure and the in vitro behavior of liposomes filled with the cytotoxic drug doxorubicin (Doxo) and functionalized on the external surface with a branched moiety containing four copies of the 8–13 neurotensin (NT) peptide is reported. The new functionalized liposomes, DOPC-$NT_4Lys(C_{18})_2$, are obtained by co-aggregation of the DOPC phospholipid with a new synthetic amphiphilic molecule, $NT_4Lys(C_{18})_2$, which contains a lysine scaffold derivatized with a lipophilic moiety and a tetra-branched hydrophilic peptide, NT8–13, a neurotensin peptide fragment well known for its ability to mimic the neurotensin peptide in receptor binding ability. Dynamic light scattering measurements indicate a value for the hydrodynamic radius (RH) of 88.3 ± 4.4 nm. The selective internalization and cytotoxicity of DOPC-$NT_4Lys(C_{18})_2$ liposomes containing Doxo, as compared to pure DOPC liposomes, were tested in HT29 human colon adenocarcinoma and TE671 human rhabdomyosarcoma cells, both of which express neurotensin receptors. Peptide-functionalized liposomes show a clear advantage in comparison to pure DOPC liposomes with regard to drug internalization in both HT29 and TE671 tumor cells: FACS analysis indicates an increase in fluorescence signal of the NT_4-liposomes, compared to the DOPC pure analogues, in both cell lines; cytotoxicity of DOPC-$NT_4Lys(C_{18})_2$-Doxo liposomes is increased four-fold with respect to DOPC-Doxo liposomes in both HT29 and TE671 cell lines. These effects could to be ascribed to the higher rate of internalization for DOPC-$NT_4Lys(C_{18})_2$-Doxo liposomes, due to stronger binding driven by a lower dissociation constant of the NT_4-liposomes that bind the membrane onto a specific protein, in contrast to DOPC liposomes, which approach the plasma membrane unselectively.

Introduction

Nanoparticles have attracted much attention for their potential application as in vivo carriers of active principles. The use of liposomes as drug carrier systems was proposed by Gregoriadis and Ryman in the early 70s.[1] These supramolecular aggregates are nontoxic, biodegradable, and non-immunogenic. Because of their size, which typically ranges in mean diameter from 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. Furthermore, liposomes exhibit preferential extravasation and accumulation at the site of solid tumors due to the increased endothelial permeability and reduced lymphatic drainage in these tissues, which has been defined as the enhanced permeability and retention effect (EPR).[2–5] The hydrophobic core of micelles and inner cavity of liposomes are carrier compartments which are able to accommodate large amount of drugs, while the shell, consisting of brush-like protective corona, stabilizes them in physiological or serum conditions and reduces toxicity of the active principle in non-target organs. Thus, associating a drug with liposomes markedly changes its pharmacokinetic and pharmacodynamic properties and lowers systemic toxicity; furthermore, the drug is prevented from early degradation and/or inactivation following introduction to the target organism.[6–9] In systemic administration, micelles or liposomes should satisfy several base requirements: high drug loading, biodegradability, long blood circulation times, slow plasma clearance, and controllable drug release profiles.

Many research efforts have been directed towards improving the safety profile of the cytotoxic anthracyclines doxorubicin, daunorubicin, and vincristine, which are associated with severe cardiotoxic side effects. For example, the alkylating agent doxorubicin acts by intercalating DNA and has been used in the liposomal formulation known as Doxil for ovarian cancer treatment. Doxil exhibited increased circulation time and decreased cardiovascular-related toxicity as compared to free doxorubicin,[10] while encapsulated doxorubicin liposomes, combined with cyclophosphamide, showed high antitumor effects in an experimental pulmonary metastatic melanoma mouse model.[11] Labeling of nanoparticles with bioactive markers that are able to direct them toward specific biological target recep-

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.201000463.

tors has led to a new generation of delivery systems for active principles.[12] Peptides and antibodies are the bioactive markers commonly used to prepare target-selective supramolecular aggregates, such as micelles and liposomes.[13–15] In particular, low-molecular-weight peptides that remain stable in vivo, well-exposed on the aggregate surface, and in appropriate conformation for binding, could be promising tools to selectively deliver nanoparticles filled with active components to the cellular target. The peptides could act to target neovascularization sites in angiogenic processes or membrane receptors overexpressed in cancer cells. Receptors for various endogenous peptides are overexpressed in several human tumors and can be used as tumor antigens.[16] In the last decade, a number of different derivatives of somatostatin, luteinizing hormone-releasing hormone (LHRH), bombesin,[17] cholecystokinin,[18] neurotensin, and neuropeptide Y[19] have been used to target tumor cells.

We have been studying the use of tetrabranched peptides (NT_4) that contain the sequence of the human regulatory peptide neurotensin (NT) as tumor targeting agents. NT is a 13 amino acid peptide originally isolated from calf hypothalamus, the full amino acid sequence is QLYENKPRRPYIL, with the C-terminus is comprised of short active fragment 8–13 (RRPYIL). NT has the dual functions of neurotransmitter or neuromodulator in the nervous system and local hormone in the periphery. NT receptor type 1 (NTS1) is overexpressed in severe malignancies such as small cell lung cancer and colon, pancreatic, and prostate carcinomas.[16,19] NT has additional well-established targets on the cell surface: NT receptor 2, NT receptor 3 (NTR3, or Sortilin), and SorLA (LR11); these latter two membrane proteins belong to the novel Vps10p-domain family.[20] Importantly, Sortilin has recently been described as having an important role in pancreatic ductal adenocarcinoma tumor cells.[21]

It is well known that peptides synthesized in a branched form not only become resistant to proteases but also increase linear peptide biological activity through multivalent binding. Using branched NT_4 fragment 8–13, conjugated to various functional units for tumor imaging and therapy,[22] we found that NT_4 conjugated to methotrexate or 5-fluoro-deoxyuridine resulted in 60% and 50% reduction, respectively,[22–24] of tumor growth in xenografted mice. Additionally, branched NT peptides have been proven to discriminate between binding of tumor versus healthy tissue in human surgical samples, validating neurotensin receptors as highly promising tumor biomarkers.[23]

Results obtained in the past for NT_4 indicated that these branched peptides are promising, novel, multifunctional, cancer-targeting molecules. The flexibility of this synthetic approach suggested it would be possible to use the branched NT on liposomal surfaces for specific drug delivery into tumor cells. In this paper, we report the structure and in vitro behavior of liposomes containing the cytotoxic drug Doxo that are functionalized on the external surface with a branched moiety containing four copies of the 8–13 NT peptide. This C-terminal NT peptide fragment is well known for its ability in mimic the NT peptide in receptor binding ability.[19]

Results and Discussion

Design and synthesis

A new amphiphilic molecule, $NT_4Lys(C_{18})_2$, (Figure 1), which contains a lysine scaffold with a lipophilic moiety and hydrophilic peptide sequences, has been designed and synthesized. Two oxoethylene (H-AhOh-OH) residues, acting as spacers, and a hydrophobic moiety based on two C_{18} alkyl chains, are linked to the lysine side chain. A branched three-lysine core, with a β-Ala spacer, is linked to the alpha amino functionality of the lysine residue; the branched core is derivatized with four copies of the 8–13 NT amino acidic sequence.

The 18-carbon lipophilic tails were selected to increase the tendency of the new amphiphilic molecule to form highly stable aggregates under physiogical conditions.[25] The branched peptide moiety, containing four copies of the 8–13 NT peptide, was selected for its well-known ability to bind NT receptors.[19] Chemical synthesis of $NT_4Lys(C_{18})_2$ was carried out on solid phase, as shown in Scheme 1, following modified protocols of the Fmoc/*t*-Bu-based procedures for solid-phase peptide synthesis (SPPS).[26] The orthogonally-protected lysine residue was anchored to the Rink amide resin. After removal of the Fmoc lysine side chain protecting group, two H-AhOH-OH linkers and the hydrophobic moiety with two C_{18} chains were sequentially introduced. Dde deprotection of the lysine N-terminal amine functionality enabled synthesis of the branched 8–13 NT tetramer according to previously reported procedures.[27] The peptide derivative was collected in good yield after HPLC–RP purification and was analyzed by mass spec-

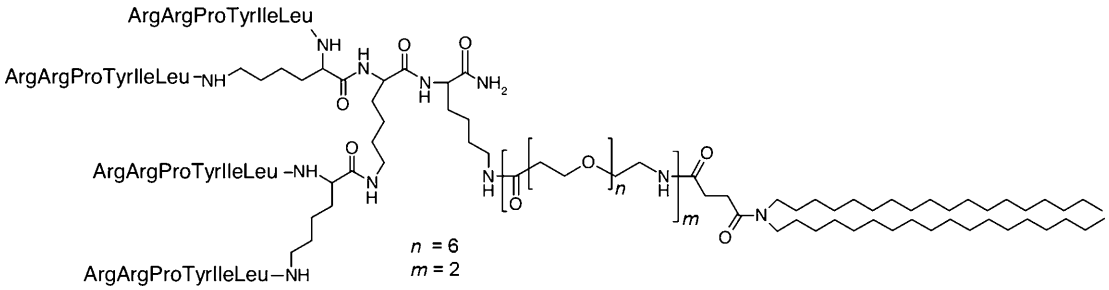
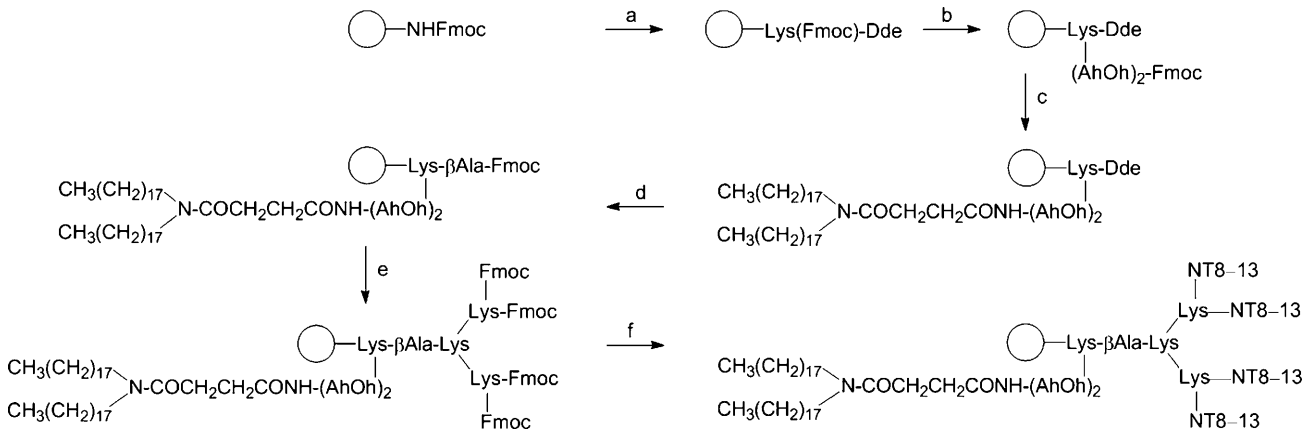


Figure 1. Chemical structure of the $NT_4Lys(C_{18})_2$ compound. Three-letter codes are used for the amino acid sequence NT8–13.



Scheme 1. Peptide synthesis according to the Fmoc solid-phase protocol. Rink amide resin is schematically represented as an empty circle. *Reagents and conditions*: a) 1. DMF/Pip (70:30), 2. Dde-Lys(Fmoc)-OH, HOBt/PyBop/DIPEA (1:1:2); b) 1. DMF/Pip (70:30), 2. Fmoc-AhOh, HOBt/PyBop/DIEPEA (1:1:2), (×2>); c) DMF/Pip (70:30), 2. *N*,*N*-dioctadecylsuccinamic acid, HOBt/PyBop/DIPEA (1:1:2); d) 1. DMF/hydrazine (98:2), 2. Fmoc-β-Ala-OH, HOBt/PyBop/DIEPEA (1:1:2); e) 1. DMF/Pip (70:30), 2. Fmoc-Lys(Fmoc)-OH, HOBt/PyBop/DIEPEA (1:1:2) (×2); f) 1. DMF/Pip (70:30), Fmoc-AA-OH, HOBt/PyBop/DIPEA (1:1:2) (×6).

trometry (MALDI-TOF) and HPLC to confirm compound identity and purity.

Aggregate formation and DLS

Mixed liposomes containing the commercial phospholipid surfactant DOPC and the synthetic amphiphile $NT_4Lys(C_{18})_2$ in a 95:5 molar ratio were prepared using known sonication and extrusion procedures.[28] Briefly, monomers were dissolved in a choloroform/methanol mixture, and the solvent was subsequently evaporated. The resulting film was hydrated in 0.1 M buffered solution (pH 7.4) at room temperature. Aggregation was successfully achieved by sonicating for 30 min and subsequent extrusion. Complete $NT_4Lys(C_{18})_2$ incorporation into DOPC liposomes was verified by analyzing a small amount of the liposomal solution using a Sephadex column. Presence in the first gel filtration fractions of a peak at 275 nm, with a UV absorbance corresponding to the Tyr residue in $NT_4Lys(C_{18})_2$, confirms the presence of an NT fragment on the liposome shell. Self-assembled DOPC liposomes were also prepared and characterized for the purpose of comparison. Dynamic light scattering measurements were taken for pure DOPC and mixed DOPC-$NT_4Lys(C_{18})_2$ aggregates in 10 mM phosphate buffer at pH 7.4. Both aggregate systems show monomodal distribution, due to the translational diffusion process, with apparent translational diffusion coefficients D. The Stokes–Einstein equation (1) is used to evaluate the hydrodynamic radius (R_H) at infinite dilution, 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 ($C = 2\times10^{-5}$ M) of the studied systems, we can approximate $D \sim D_0$, and Equation (1) can reasonably be used to estimate the R_H of the aggregates.

$$R_H = \frac{K_B T}{6\pi r D_0} \quad (1)$$

The R_H values found for DOPC and DOPC-$NT_4Lys(C_{18})_2$ liposomes are 90.0 ± 8.1 and 88.3 ± 4.4 nm, respectively. The radius found for the DOPC liposome is in good agreement with literature data.[29] Mixed liposomes in which a small amount (5%) of NT peptide derivative is added to the DOPC phospholipid have similar liposomal structure and dimension to pure DOPC liposomes, as already demonstrated for pure and mixed aggregates based on similar amphiphilic monomers.[28]

Doxorubicin loading and release

Doxorubicin (Doxo) was loaded into the liposome using the pH gradient method.[30] Liposomes were prepared at pH 4.0, and phosphate buffer was diluted to 2.5 mM because of the poor solubility of doxorubicin in phosphate solution with the pH range 5.0–8.5.[31] The Doxo loading content was calculated by fluorescence measurements with subtraction of the amount of free doxorubicin, eluted by gel filtration, from the total amount of initial doxorubicin. The Doxo loading content value (DLC), defined as the weight ratio of the encapsulated Doxo versus the amount of the amphiphilic moieties, was 0.065.

Unloaded doxorubicin was removed using a Sephadex G50 column. The release profile of Doxo from liposomes was studied within 96 h using a dialysis membrane immersed in cell medium at 37 °C. Transfer of released Doxo through the dialysis membrane to buffer solution was assumed to occur rapidly, and the release of Doxo from its liposomal vehicle to medium was assumed to be the rate-limiting step in this process. The amount of Doxo released was estimated by UV/vis spectroscopy at 480 nm, and the release profiles for DOPC-$NT_4Lys(C_{18})_2$-Doxo liposomes are reported in Figure 2 as a percentage of the total released Doxo as a function of time. As expected, DOPC-Doxo liposomes do not show any significant differences as compared to DOPC-$NT_4Lys(C_{18})_2$-Doxo liposomes (data not shown). After 8 h, corresponding to the incubation time of the cells in the presence of Doxo-containing liposomes, approximately 25% of Doxo was released from mixed liposomes,

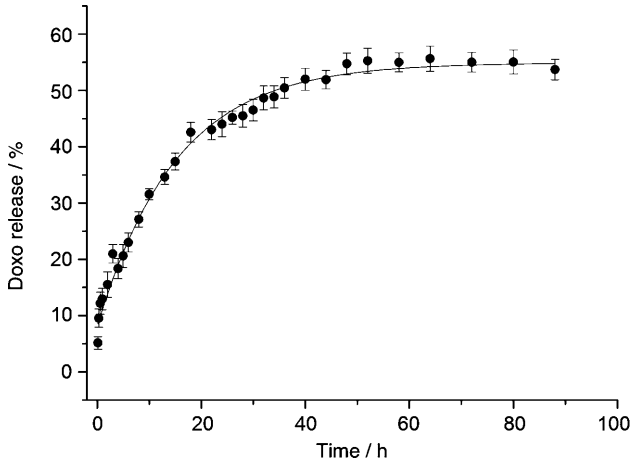


Figure 2. Release of Doxo by DOPC-$NT_4Lys(C_{18})_4$-Doxo liposomes (●). The amount of Doxo released was estimated by UV/vis spectroscopy at 480 nm.

while $t_{1/2}$ (time at which 50% of Doxo had leaked from liposomes) was 35 h. These results are in agreement with data previously reported by Allen et al.[32] of Doxo release from PC/Chol/DSPE-Peg 2000 (2:1:0.1) liposomes in which $t_{1/2}$ ranged from 11.2–85.5 h, depending on the phosphatidylcholine (PC) used in liposome formation.

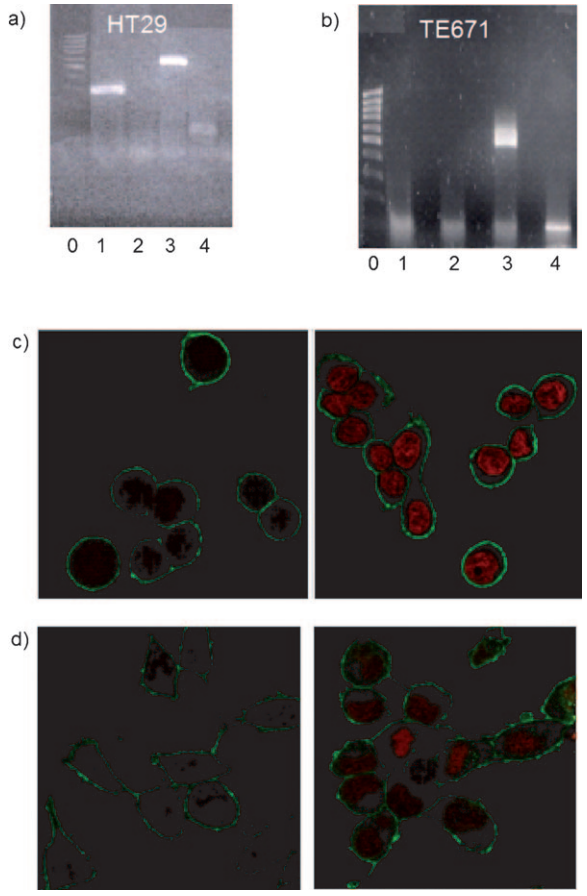


Figure 3. RT-PCR expression of NT receptors in a) HT29 and b) TE671 cells, lane 0 = DNA standard, lane 1 = NTR1, lane 2 = NTR2, lane 3 = NTR3, lane 4 = beta 2 microglobulin. Confocal microscopy: c) HT29 and d) TE671 cells were incubated with DOPC-$NT_4Lys(C_{18})_2$-Doxo liposomes (200 nM, right) and with DOPC-Doxo liposomes (200 nM, left) for 2 h at 37 °C. Plasma membrane was stained with lectin-FITC (green).

Cell internalization of DOPC-NT4Lys(C18)2-Doxo liposomes

The selective internalization of NT_4-derivatized liposomes, compared to nude liposomes, was tested in HT29 human colon adenocarcinoma and TE671 human rhabdomyosarcoma. The HT29 cell line expresses two NT receptors, NTR1 and NTR3, while TE671 only expresses NTR3, as confirmed by RT-PCR (Figure 3a and b). Internalization was studied by confocal microscopy following the red fluorescence signal of Doxo (Figure 2c and d). Cells were incubated at a number of temperatures (4, 25, and 37 °C) for various time intervals (from 30 min–3 h). The concentrations of liposomes, (200 nM, 400 nM, and 1 μM) were calculated as the molarity of Doxo. Figure 3 is representative and shows images taken after 2 h incubation with 200 nM liposomes at 37 °C. At lower temperatures and all concentrations, the internalization of both types of liposomes is slightly lower, as expected (see Supporting Information). The internalization process is near completion by 30 min at all temperatures and concentrations. The intracellular red fluorescence was very strong for cells incubated with DOPC-$NT_4Lys(C_{18})_2$-Doxo liposomes, whereas nonfunctionalized liposomes gave very faint signals under the same experimental conditions. A comparison between functionalized and nude liposomes suggests an important net advantage obtained through conjugation of the particles with branched NT.

Cytotoxicity of DOPC-$NT_4Lys(C_{18})_2$-Doxo liposomes

HT29 and TE671 cells were incubated with various concentrations, from 8 nM–25 μM, of DOPC-$NT_4Lys(C_{18})_2$-Doxo liposomes and DOPC-Doxo liposomes. After 8 h incubation, cells were washed and incubated for six days. Washing was performed to avoid diffusion of free Doxo from the liposomes during the six day incubation period. As reported in Figure 4, DOPC-$NT_4Lys(C_{18})_2$-Doxo liposomes showed an EC_{50} value of 1.30 μM in HT29, whereas the EC_{50} value of DOPC-Doxo liposomes was 5.48 μM; therefore, the labeling of liposomes with a tumor selective moiety produced a four-fold increase in activity ($p < 0.05$). The same increase in cytotoxicity was observed in TE671. The cytotoxic behavior of free Doxo and empty liposomes under the same experimental conditions (see Supporting Information) was also investigated. As expected, Doxo cytotoxicity is markedly higher with respect to free versus encapsulated drug ($EC_{50} = 0.323$ μM), while no cytotoxicity was observed for empty DOPC liposomes when they are studied in the 5 nM–400 μM concentration range.

FACS analysis

HT29 and TE671 cells were incubated with various concentrations (from 500 nM–10 μM) of either DOPC-$NT_4Lys(C_{18})_2$-Doxo liposomes or DOPC-Doxo liposomes (Figure 5). At the highest concentration, the difference between the two types of lipo-

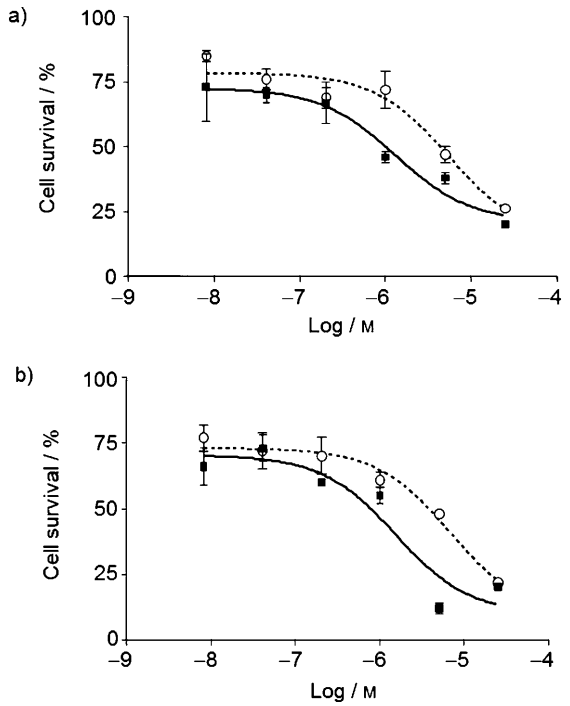


Figure 4. Cytotoxicity: a) HT29 and b) TE671 cells were incubated with various concentrations, from 8 nM–25 μM, of DOPC-$NT_4Lys(C_{18})_2$-Doxo liposomes (■) and DOPC-Doxo liposomes (○). After 8 h incubation, cells were washed and incubated for six days. Percentage of cell survival is calculated in comparison to untreated controls. EC_{50} values: DOPC-$NT_4Lys(C_{18})_2$-Doxo liposomes EC_{50} = 1.81 μM (HT29), EC_{50} = 1.53 μM (TE671); DOPC-Doxo liposomes EC_{50} = 5.48 μM (HT29), EC_{50} = 6.72 μM (TE671).

somes was maximized. In HT29, DOPC-$NT_4Lys(C18)_2$-Doxo liposomes gave an increase in fluorescent signal of 40% compared to the analogous DOPC-Doxo liposomes. In TE671 (not shown), the NT_4-derivatized liposomes showed a 20% increase in fluorescence with respect to the nude analogues.

Conclusions

In vitro results achieved thus far indicate very promising performance of liposomes labeled with branched NT, which may be used for selective targeting of tumor cells. Functionalized liposomes show a clear advantage in comparison to native liposomes in tumor cell drug internalization, both in HT29 and TE671 cells. Cytotoxicity of DOPC-$NT_4Lys(C_{18})_2$-Doxo liposomes is increased four-fold with respect to DOPC-Doxo liposomes in both HT29 and TE671 cell lines. Results from FACS analysis are in line with these results, resulting in a fluorescence signal increase of the NT_4 liposomes as compared to the nude analogues in both cell lines. All of these effects can be ascribed to the fact that DOPC-$NT_4Lys(C_{18})_2$-Doxo liposomes have a higher rate of internalization, due to stronger binding driven by a lower dissociation constant of the NT_4 liposomes that bind the membrane onto a specific protein, in contrast to the nude liposomes, which approach the plasma membrane unselectively. These in vitro results can be considered very promising, in the sense that the possibility to derivatize liposomes in order to make them selective carriers is considered an endpoint in this field. Selective liposomes can then be loaded with any single chemotherapeutic or a combination of drugs to become a valuable weapon to fight cancer.

Experimental Section

Chemistry

Materials and instrumentation: Fmoc-protected amino acid derivatives, coupling reagents, and Rink amide *p*-methylbenzhydrylamine MBHA 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). *N,N*-Dioctadecylsuccinamic acid was synthesized according to a published procedure.[33] All other chemicals were commercially available by Sigma–Aldrich, Fluka (Buchs, Switzerland), or LabScan (Stillorgan, Dublin, Ireland) and were used as received, unless otherwise stated. Preparative HPLC was carried out on a LC8 Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a UV lambda-Max Model 481 detector using a Phenomene C_4 column (300 Å, 250×21.20 mm, 5 μ; Torrance, CA), eluting with A) water/0.1% trifluoroacetic acid (TFA) and B) CH_3CN/0.1% TFA from 40–95% over 25 min at a 20 mL min^{-1} flow rate. Analytical HPLC was carried out using a Jupiter C4 column (300 Å, 150×4.60 mm, 5 μ) and the same gradient at a 1 mL min^{-1} flow rate. Fluorescence spectra were recorded on a Jasco Model FP-750 spectrofluorimeter, using a 1.0 cm path length quartz cell. UV measurements were performed on a UV/vis Jasco V-5505 spectrophotometer equipped with a Jasco ETC-505T Peltier temperature controller with a 1 cm quartz cuvette (Hellma). (8*S*,10*S*)-10-(4-amino-5-hydroxy-6-methyl-tetrahydro-2*H*-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. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). The liposomes were extruded using a mini-extruder purchased from Avanti Polar Lipids. Dynamic light scattering measurements were carried out by mini-DAWN TREOS Wyatt Techology (Santa Barbara, CA, USA). Mass spectra were recorded on a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) Voyager-DE (Perseptive Biosystems).

Synthesis of (Arg-Arg-Pro-Tyr-Ile-Leu)$_4$-(Lys)$_2$-Lys-βAla-Lys-(AhOH)$_2$-(C_{18})$_2$ ($NT_4Lys(C_{18})_2$): The monomer was synthesized on solid phase using Rink amide (MBHA) resin (0.54 mmol g^{-1}; 0.048 mmol, 0.090 g) as polymeric support. After swelling of the resin in 2.0 mL of *N,N*-dimethylformamide (DMF for 1 h, the Fmoc protecting group was removed by a mixture of piperidine/DMF (30:70). The carboxylic group of Dde-Lys(Fmoc)-OH (0.100 mmol, 0.532 g) was activated by 1.0 equiv of benzotriazol-1-yl-oxytris(pyrrolidino)phosphonium (PyBop), 1-hydroxybenzotriazole (HOBt), and 2.0 equiv of *N,N*-diisopropylethylamine (DIPEA) in DMF. The solution was added to the resin, and the slurry suspension was stirred for 1 h. Coupling of the lysine residue was performed twice and checked by the Kaiser colorimetric test. The solution was filtered and the resin washed with three portions of DMF and three portions of CH_2Cl_2. After the removal of the Fmoc protecting group from the lysine side chain, two molecules of Fmoc-Ahoh-OH were sequentially coupled according to previously described coupling and deprotection conditions. After Fmoc deprotection, *N,N*-dioctadecylsuccinamic acid (0.1 mmol, 0.62 g) was condensed, with 2.0 equiv dissolved in DMF/CH_2Cl_2 (50:50). The lipophilic moiety was activated in situ by the standard HOBt/PyBop/DIPEA procedure, and the coupling reaction proceeded for 1 h. The resin was

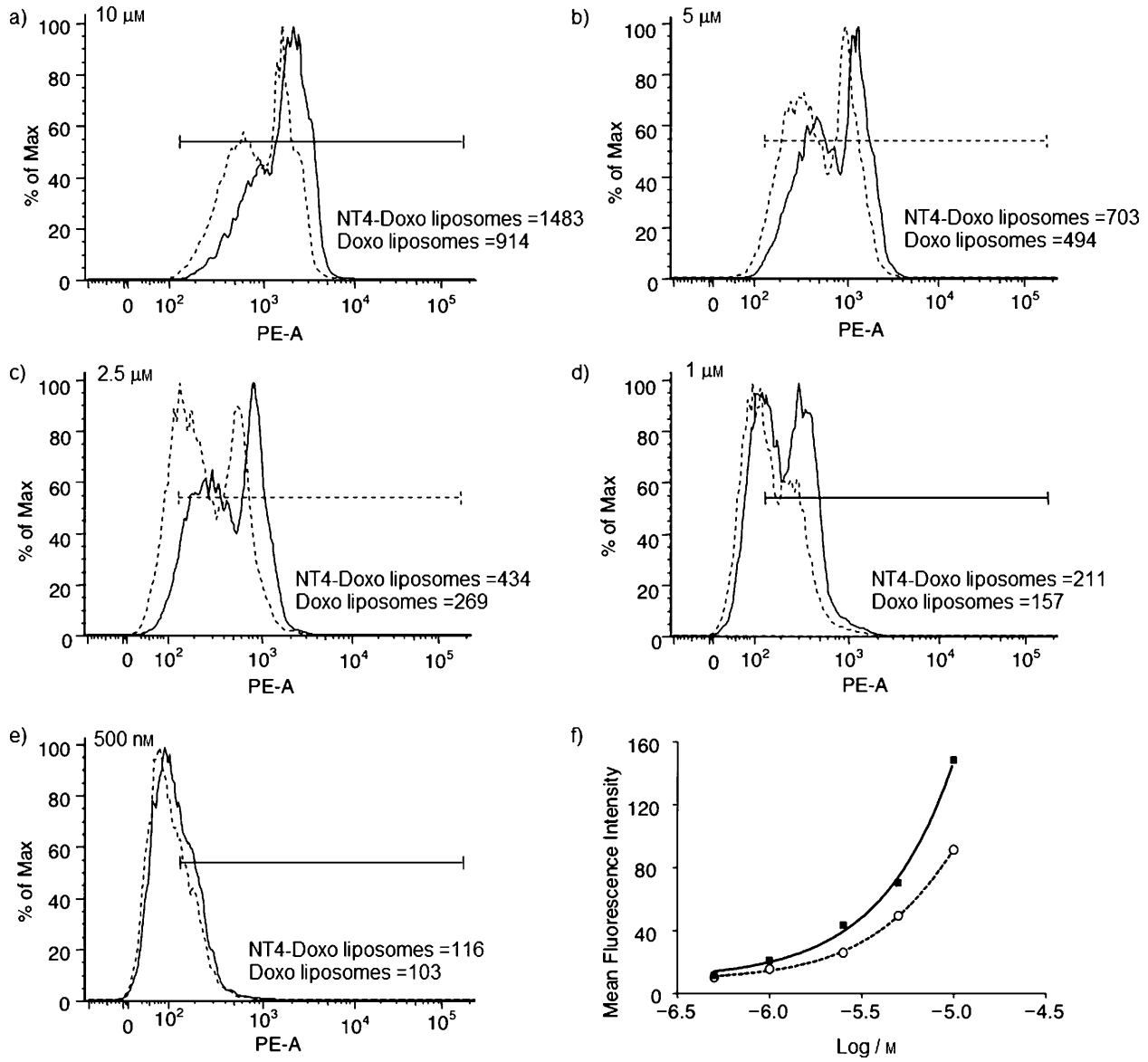


Figure 5. FACS analysis: HT29 cells were incubated with NT_4-Doxo liposomes (——) and with Doxo liposomes (-----). Cells were fixed for 10 min at room temperature with 4% PFA-TBS and then incubated for 1 h and 0.5 h at room temperature with various concentrations of liposomes (a) 10 μM; b) 5 μM; c) 2.5 μM; d) 1 μM; e) 500 nM). f) Fluorescent signals were measured with a BD FACSCanto II and analyzed by nonlinear regression. Differences between NT_4-Doxo liposomes (■) and Doxo liposomes (○) are shown.

washed three times with DMF, then, 1-(4,4-dimethyl-2,6-dioxo-cyclohexylidene)3-methylbutyl (Dde) was removed from the N-terminal amine functionality of the lysine residue by treatment with DMF/hydrazine (98:2). The peptide–resin was stirred with 3.0 mL of this solution for 10 min. The treatment was repeated twice, and the deprotection reaction was monitored by the qualitative Kaiser test and UV spectroscopy. The following amino acid derivatives were coupled sequentially to the free N-terminal amine functionality using the previous described coupling and deprotection conditions: Fmoc-βAla-OH, Fmoc-Lys(Fmoc)-OH, Fmoc-Lys(Fmoc)-OH, Fmoc-Leu-OH, Fmoc-Ile-OH, Fmoc-Tyr(*t*Bu)-OH, Fmoc-Pro-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH. Finally, the N-terminal Fmoc protecting group was removed, and the amphiphilic peptide was cleaved with TFA containing triisopropylsylane (2.5%), and water (2.0%) over a period of 24 h. The peptide product was precipitated by adding water dropwise at 0 °C, purified by HPLC, and lyophilized. The final product ($NT_4Lys(C_{18})_2$) was obtained in 25% yield and analyzed by HPLC and MALDI-TOF mass spectroscopy: HPLC: $t_R = 39.2$ min; MALDI-TOF: MW = 5068 Da.

Aggregate 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 PHM 220. In most cases, the samples to be measured were prepared from stock solutions. Mixed aggregates of DOPC and $NT_4Lys(C_{18})_2$ (95:5 molar ratios) were prepared by dissolving the two amphiphiles in a small amount of MeOH/$CHCl_3$ (50:50), subsequently evaporating the solvent by slowly rotating the tube containing the solution under a stream of nitrogen. In this manner, a thin film of amphiphiles was obtained which was hydrated by addition of 0.1 M phosphate buffer (pH 7.4) and sonicated for 30 min. All samples were extruded 21 times through a polycarbonate membrane (100 nm pore size). A similar procedure was used to prepare pure

DOPC liposomes. All solutions were then diluted with phosphate buffer to a final lipid concentration of 2.0×10^{-5} M. Samples were centrifuged at 13,000 rpm for 5 min at room temperature, and the measurement was performed at 25 °C. Scattered light intensities were measured at a fixed scattering angle ($\theta = 90°$). Results were processed with the Qels program (Wyatt Technology).

Doxorubicin loading: DOPC-Doxo and DOPC-$NT_4Lys(C_{18})_2$-Doxo liposomal formulations were prepared by loading doxorubicin·HCl into DOPC and DOPC-$NT_4Lys(C_{18})_2$ supramolecular aggregates, respectively. Doxo was loaded using the pH gradient method with free Doxo removed by gel filtration. Briefly, the liposomal solution was prepared as reported above 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, 210 μL of 2.36×10^{-3} M Doxo solution in 2.5 mM phosphate buffer were added to 100 μL of liposomal solution. This 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 or fluorescence emission at 590 nm. Emission spectra were recorded at room temperature. Equal excitation and emission bandwidths were used throughout experiments, with a recording speed of 125 nm min^{-1} and automatic selection of the time constant. Subsequently, unloaded Doxo was removed using a Sephadex G50 column pre-equilibrated with 2.5 mM phosphate buffer at pH 7.4. The Doxo 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.

Doxorubicin release: In vitro doxorubicin release from DOPC and DOPC-$NT_4Lys(C_{18})_2$ liposomes was measured using a dialysis method. Before starting the release experiment, Doxo-loaded liposomes underwent two ultracentrifugations at 34000 rpm for 3.5 h at 4 °C using the Beckman SW55 rotor to remove free doxorubicin from the solution. Doxo-loaded liposomes were redissolved in water solution. 1.5 mL of Doxo-containing aggregates were added to a dialysis bag (MW cutoff 3500), placed into 15 mL of cell media, and incubated while stirring for 96 h at 37 °C. Aliquots (2 mL) of the dialyzed solution was removed at various time points to measure drug content in the dialyzed solution and was replaced immediately by the same volume of fresh medium. The concentration of released Doxo was analyzed by UV/vis spectroscopy at 480 nm.

Biology

Cell cultures: HT29 human colon adenocarcinoma cells were grown in the recommended medium, McCoy's 5A, supplemented with 10% fetal calf serum, 200 μg mL^{-1} glutamine, 100 μg mL^{-1} streptomycin, and 60 μg mL^{-1} penicillin and maintained at 37 °C in 5% CO_2. Cell lines were purchased from Istituto Zooprofilattico Sperimentale (Brescia, Italy).

Reverse transcription PCR: Reverse transcription PCR was used for analysis of mRNA. Total RNA was isolated using an RNeasy kit (Qiagen) with 3106 cells, as suggested by the manufacturer. One step reverse transcription PCR (Qiagen) was applied for retrostranscription and cDNA amplification of NTR1, NTR2, and NTR3. The oligonucleotides used as primers are given below with the predicted sizes of amplified fragments 291 bp, 429 bp, 552 bp, and 114 bp for human NTR1, NTR2, NTR3, and beta 2 microglobulin, respectively. NTR1 sense: TCATCGCCTTTGTGGTCTGCT, antisense: TGGTTGCTGGACACGCTGTCG; NTR2 sense: GTCTCCTCAGCTTCATCGTAT, antisense: TCCCCAAAGCCTGAAGCTGTA; NTR3 sense: AGAATGGTCGAGACTATGTTG, antisense: AAGAGCTATTCCAAGAGGTCC; beta 2 microglobulin sense: ACCCCCACTGAAAAAGATGA, antisense ATCTTCAAACCTCCATGATG.

Cell binding and internalization: HT29 and TE671, plated at a density of 25000 cells per well and incubated overnight, were fixed for 10 min in 4% *para*-formaldehyde (PFA)/Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.4) and incubated at a number of temperatures (4, 25, and 37 °C) for various time intervals (30 min–3 h) with liposomes (200 nM Doxo) diluted in 0.3% bovine serum albumin (BSA)/TBS. Cells were then washed three times with TBS and the membranes stained with lectin/FITC 0.5 μg mL^{-1} in 0.3% BSA/TBS for 15 min at room temperature. Images were taken with a TCS SP5 Leica confocal microscope.

Cytotoxicity of drug-conjugated branched NT: HT29 and TE671 cells were plated at a density of 25000 cells per well in 96-well microplates. After 24 h, cells were exposed to various concentrations of liposomes (8 nM–25 μM). After 8 h incubation, cells were washed and grown for six days at 37 °C in the same medium. Growth inhibition was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). EC_{50} values were calculated by non-linear regression analysis using GraphPad Prism software (v. 3.02). Values obtained from the untreated controls resulted in 100% cell viability.

FACS analysis: HT29 cells and TE671 cells were incubated with DOPC-Doxo and DOPC-$NT_4Lys(C_{18})_2$-Doxo liposomes. 100000 cells per well were fixed for 10 min at room temperature with 4% PFA-TBS, then incubated in 96-well U-bottomed plates for 0.5 and 1 h at room temperature with various concentrations of liposomes (500 nM–10 μM) in 5 mM TBS-EDTA with 0.5% BSA. Flow cytometry measurements were obtained by analyzing 10000 events with a FACSCanto II (BD, Franklin Lakes, NJ, USA). Results were analyzed by nonlinear regression analysis using GraphPad Prism software.

Acknowledgements

The authors thank the Italian Minister of Health for financial support of the projects New molecular entity for diagnosis and therapy in Oncology and MIUR (FIRB Proj. RBRN07BMCT), as well as the Italian Association for Cancer Research (AIRC), Istituto Toscano Tumori, and Fondazione Monte dei Paschi di Siena.

Keywords: branched neurotensin peptides · liposomal doxorubicin · selective drug delivery · supramolecular aggregates

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Received: October 26, 2011
Revised: January 10, 2011
Published online on ■■ ■■, 2011

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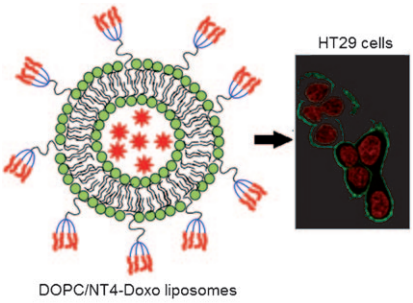

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Target-Selective Drug Delivery through Liposomes Labeled with Oligobranched Neurotensin Peptides

