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RESEARCH ARTICLE

Bombesin peptide antagonist for target-selective delivery of liposomal doxorubicin on cancer cells

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Abstract

Purpose: This study addresses novel peptide modified liposomal doxorubicin to specifically target tissues overexpressing bombesin (BN) receptors.

Methods: *DOTA-(AEEA)$_2$-peptides* containing the [7–14]bombesin and the new BN-AA1 sequence have been synthesized to compare their binding properties and in serum stabilities. The amphiphilic peptide derivative (*Mon*Y-BN-AA1) containing BN-AA1, a hydrophobic moiety, polyethylenglycole (PEG), and diethylenetriaminepentaacetate (DTPA), has been synthesized. Liposomes have been obtained by mixing of *Mon*Y-BN-AA1 with 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC).

Results: Both ^{111}In labeled peptide derivatives present nanomolar Kd to PC-3 cells. ^{177}Lu labeled peptide *DOTA-(AEEA)$_2$-BN-AA1* is very stable (half-life 414.1 h), while *DOTA-(AEEA)$_2$-BN*, shows a half-life of 15.5 h. *In vivo* studies on the therapeutic efficacy of DSPC/*Mon*Y-BN-AA1/Dox in comparison to DSPC/*Mon*Y-BN/Dox, were performed in PC-3 xenograft bearing mice. Both formulations showed similar tumor growth inhibition (TGI) compared to control animals treated with non-targeted DSPC/Dox liposomes or saline solution. For DSPC/*Mon*Y-BN-AA1/Dox the maximum effect was observed 19 days after treatment.

Conclusions: DSPC/*Mon*Y-BN-AA1/Dox nanovectors confirm the ability to selectively target and provide therapeutic efficacy in mice. The lack of receptor activation and possible acute biological side effects provided by using the AA1 antagonist bombesin sequence should provide safe working conditions for further development of this class of drug delivery vehicles.

Keywords: Liposomes for drug delivery, bombesin peptide, doxorubicin delivery, anticancer efficacy, animal studies

Introduction

The Bombesin receptor family is a well-known important target for diagnostic and therapeutic applications. It consists of four receptor subtypes including the neuromedin B receptor (BB1), the gastrin-releasing peptide receptor (GRP-R, BB2), the orphan receptor subtype (BB3), and the amphibian receptor (BB4) (Battey et al., 1991; Fathi et al., 1993; Nagalla et al., 1995; Wada et al., 1991). The first two are widely diffused in normal tissues and on cells of several human tumors: for example, the GRP-R has been found overexpressed by tumor cell lines of ovarian cancers, breast cancers and prostate cancers (Markwalder & Reubi, 1999; Gugger & Reubi, 1999; Fleischmann et al., 2007), while BB1 has been found overexpressed in ileal

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carcinoids. They bind the mammalian bombesin peptides: gastrin-releasing peptide (GRP) and neuromedin B (NMB). Both peptides demonstrate a broad spectrum of pharmacological and biological responses and affect the growth and/or differentiation of a number of important human tumors including colon, prostate, lung and some gynecologic cancers.

The fourteen-residue Bombesin peptide (BN), its eight-residues C-terminal peptide sequence ([*7–14*]BN), and many other Bombesin analogues acting as agonist or antagonists, have been modified to selectively carry diagnostic or therapeutic agents to their receptors. Many studies demonstrate that bombesin fragment [*7–14*] BN modified on the N-terminus with radioactive metal complexes of 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) or diethylenetriaminepenta acetic acid (DTPA) preserves its affinity for these receptors and several BN analogues containing aliphatic or amino acidic linkers placed between the BN peptide and the radioactive metal complex, display selective uptake on cells overexpressing GRP receptors, with IC_{50} values in the nanomolar range, in *in vitro* experiments and in *in vivo* studies (Smith et al., 2005; Rogers et al., 2003; Parry et al., 2007).

As already reported for other neuroendocrine peptides (CCK8, RGD, Octreotide), bombesin peptides can be not only covalently bound to a diagnostic or a therapeutic agent, but also coupled to nanovectors, such as nanoparticles or liposomes, to deliver contrast agents (Accardo et al., 2010; Hosta-Rigau et al., 2010; Lee et al., 2010; Mendoza-Sánchez et al., 2010; Martin et al., 2009) and/or cytotoxic drugs (Chanda et al., 2010). In order to achieve this last goal, the bioactive peptide must maintain its high affinity for the target receptor when exposed on the external surface of the nanovector and should remain stable in the blood stream for the prolonged time in which the cargo vehicle remains in circulation. Moreover, to avoid possible side-effects induced by the injection of large quantities of nanovectors, and consequently bioactive peptides, it is desirable that the bioactive peptide behave as an antagonist in order to avoid activation of biological pathways (Chan et al., 2011; Zhu & Chen, 2004).

With the aim of developing new target selective theranostic agents against cells overexpressing bombesin receptors, a new class of liposomes, based on a mix of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) phospholipid with a new synthetic amphiphilic monomer, *Mon*Y-BN, and encapsulating the cytotoxic drug doxorubicin (Dox), were recently developed (Accardo et al., 2012). *Mon*Y-BN consists on a monomer containing the [*7–14*]Bombesin peptide fragment, the DTPA chelating agent, the hydrophobic moiety with two C18 alkyl chains and PEG spacers all in a single molecule (Accardo et al., 2012). This system showed encouraging results *in vivo*, leading to a significant tumor growth inhibition, compared to "conventional" liposomes encapsulating Dox (60% vs 36%), in PC-3 xenograft-bearing mice.

In the current manuscript, we attempt to enhance "performance" of the nanovectors by utilizing a modified sequence of the [7–14]Bombesin peptide that should improve the overall properties of the agent. The new peptide sequence that has been introduced into the *Mon*Y monomer, should afford higher serum stability, maintain high receptor affinity and is expected to behave as a bombesin antagonist, thus providing more desirable *in vivo* properties of the entire nanovector.

Methods

Instrumentation

Preparative HPLCs were carried out on a LC8 Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a UV lambda-Max Model 481 detector. Crude products were eluted with H_2O/0.1% trifluoroacetic acid (TFA) (A) and CH3CN/0.1% TFA (B) at 20 mL/min flow rate. Purifications of peptide conjugates were performed by using. Purifications of lipophilic peptides were performed by using a Phenomenex (Torrance, CA) C4 (300 Å, 250 × 21.20 mm, 5 µ) column eluted with an H_2O/0.1% trifluoroacetic acid (TFA) (A) and CH_3CN/0.1% TFA (B) from 20% to 95% over 25 min at 20 mL/min flow rate. LC–MS analyses were performed by using Finnigan Surveyor MSQ single quadrupole electrospray ionization (Finnigan/Thermo Electron Corporation San Jose, CA). 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).

Pharmaceuticals and reagents

Protected Nα-Fmoc-amino acid derivatives, coupling reagents and Rink amide MBHA resin were purchased from Calbio-chem-Novabiochem (Laufelfingen, Switzerland). The Fmoc-21-amino-4,7,10,13,16,19-hexaoxaheneicosanoic acid (Fmoc-Ahoh-OH) and Fmoc-8-amino-3,6-dioxaoctanoic acid (Fmoc-AdOO-OH) were purchased from Neosystem (Strasbourg, France). N,N-dioctadecylsuccinamic acid was prepared according to the experimental procedure reported in literature (Schmitt & Dietrich, 2004). α-(9-Fluorenylmethyloxycarbonyl) amino-ω-carboxy poly(ethylene glycol) (Fmoc-NH-Peg27-COOH) was purchased by Iris Biotech GmbH (Marktredwitz, Germany). DOTA(OtBu)3-OH (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate tert-butyl ester)) and DTPA(OtBu)4-OH (diethylenetriaminepentaacetate tetra-tert-butyl ester) was purchased from Chematech (Dijon, France). Citrate acid, sodium citrate, sodium chloride and Doxorubicin were obtained from Sigma Chemical Co. (St. Louis Mo, USA). All other chemicals were commercially available by Sigma-Aldrich, Fluka (Bucks, Switzerland) or LabScan (Stillorgan, Dublin, Ireland) and were used as received unless otherwise stated. 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) was purchased from Lipoid GmbH (Cam,

Switzerland). All solutions were prepared by weight using doubly distilled water.

Peptide derivatives synthesis

DOTA-(AEEA)$_2$-BN and *DOTA-(AEEA)$_2$-BN-AA1*

Peptide syntheses were carried out in solid-phase under standard Fmoc strategy, by using 433A Applied Biosystems automatic synthesizer. Rink-amide MBHA resin (0.78 mmol/g, 0.5 mmol scale, 0.640 g) was used. The elongations of the bombesin peptides were achieved by sequential addition of Fmoc-AA-OH with benzotriazol-1-yl-oxy-tripyrrolidinophosphonium hexafluorophosphate/1-hydroxy-1,2,3-benzotriazole/N,N-diisopropylethylamine (PyBOP/HOBt/DIPEA) (1:1:2) as coupling reagents, in N,N-dimethylformamide (DMF) in pre-activation mode. The mixture was stirred for 1 h and after filtration. All couplings were performed twice for 1 h, by using an excess of 4 equivalents for the single amino acid derivative. Fmoc deprotections were obtained by 30% solution of piperidine in DMF. When the bombesin sequences were complete, the Fmoc N-terminal protecting group was removed and two residues of Fmoc-AdOO-OH were added. An excess of 2 equivalents of Fmoc-AdOO-OH, PyBop and HOBt and 4 equivalents of DIPEA were dissolved in DMF and added to the manual vessel. When the linkers were coupled on the peptide chain, the DOTA-tetraester chelating agent was linked, through its free carboxyl function. This coupling step was performed using 2.0 equivalents of DTPA tetraester and HATU, and 4 equivalents of DIPEA in DMF as solvent. The coupling time, compared with the classical solid phase peptide synthesis protocol, was increased up to 2 h. For deprotection and cleavage, the fully protected resins were treated with a mixture of trifluoroacetic acid (TFA), triisopropylsilane (TIS), and water in the TFA/TIS/H_2O 95/2.5/2.5 ratio, and the free product precipitated at 0°C by adding water drop wise. Purification of the crude mixture was carried out by RP-HPLC (λ = 280 nm). The final product was recovered at purity higher than 95% and with a final yields of 50%. Mass spectra confirm the product identity (see Table 1).

DOTA-(AEEA)$_2$-BN: Rt =12.91 MW: 1613 amu MS (ESI$^+$): m/z (%): [M+2H$^+$]/2 = 807.5 amu

DOTA-(AEEA)$_2$-BN-AA1: Rt = 13.13 MW: 1976 amu MS (ESI$^+$): m/z (%): [M+2H$^+$]/2 = 989 amu

Synthesis of $(C_{18}H_{37})_2NCO(CH_2)_2CO$-Peg27-Lys(DTPA)-Ahoh-DPhe-Gln-Trp-Ala-Val-NMeGly-His-Sta-Leu-Amide; (*Mon*Y-BN-AA1)

Synthesis of *Mon*Y-BN-AA1 was carried out in solid-phase under standard Fmoc/tBu strategy, as previously described (Chang & White, 2000) for the parent *Mon*Y-BN monomer. Rink-amide MBHA resin (0.78 mmol/g, 0.5 mmol scale, 0.640 g) was used as solid support. Peptide derivative was cleaved from the resin with TFA (5 mL) containing 2.5% (v/v) water and 2.0% (v/v) tri-isopropylsylane (TIS) as a scavenger at room temperature for 2 h. Free peptide was precipitated in cold ethyl ether and lyophilized from a 50% H_2O/CH_3CN solution. Crude peptide was purified by reversed phase HPLC and each fraction was characterized by LC–MS analysis.

$(C_{18}H_{37})_2NCO(CH_2)_2CO$-Peg27-Lys(DTPA)-Ahoh-DPhe-Gln-Trp-Ala-Val-NMeGly-His-Sta-Leu-Amide; *Mon*Y-BN-AA1: Rt = 25.2 min; (MW = 3870) [M+3H$^+$]/3 = 1291 amu and [M+4H$^+$]/4 = 968.5 amu.

Serum stability

^{177}Lu-DOTA-peptide conjugates were prepared by dissolving 10 μg of peptide in 250 μL of sodium acetate buffer (0.4 M, pH 5.0) and incubating the peptide solution with $^{177}LuCl_3$ (74–185 MBq) for 30 min at 95°C. Quality control was performed using radio-HPLC. To 1.0 mL of freshly prepared human serum, previously equilibrated in a 5% CO_2 environment at 37°C, we added 0.6 nmol of the ^{177}Lu-labeled peptide. The mixture was incubated in a 5% CO_2, 37°C environment. At different time points, 100 μL aliquots (in triplicate) were removed and treated with 200 μL of EtOH to precipitate serum proteins. Samples were then centrifuged for 15 min at 500 rpm. 50 μL of supernatant were removed for activity counting in a γ-well counter, the sediment was washed twice with 1 mL of EtOH and counted, and the activity in the supernatant was compared with the activity in the pellet to give the percentage of peptides not bound to proteins or radiometal transferred to serum proteins. The supernatant was analyzed by radio-HPLC (eluents: A = 0.1% trifluoroacetic acid in water and B = acetonitrile; gradient: 0–25 min 95–50% B) to determine the relative amount of intact peptide and its metabolites in serum. The half-life was calculated fitting a first order reaction to the experimental data (Equation 1) using Prism software (GraphPad Software Inc.).

$$[A] = e^{-k_1 t} \quad (1)$$

Saturation binding experiments

The peptide conjugates were studied *in vitro* by performing cellular binding-saturation experiments using increasing concentrations of the ^{111}In-DOTA-peptide ranging from 0.5 to 50 nM. Confluent PC-3 cells were seeded in six-well plates (~$1.0 \cdot 10^6$ cells) 24 h before starting the experiments. For blocking experiments excess

Table 1. LC-MS data, dissociation constant (K_d), apparent number of binding sites per cells (B_{max}) and half-life of DOTA-(AEEA)$_2$-BN and DOTA-(AEEA)$_2$-BN-AA1 peptide conjugates.

Peptide conjugates	Rt/min	Mw/amu	K_d/nM	B_{max} (binding sites/cell)	half-life/h
DOTA-(AEEA)$_2$-BN	12.91	1613	3.30 ± 0.39	2.45×0^5 ± 10.07	15.5 ± 2.1
DOTA-(AEEA)$_2$-BN-AA1	13.13	1796	5.91 ± 0.57	3.52×0^5 ± 10.10	414.1 ± 39.4

(1000 fold) of cold peptide was used. For each radioligand, triplicates were prepared for every concentration, for both total binding and nonspecific binding. Before adding the radioligands to the wells, the plates were placed on ice for 30 min. After adding the radioligands and the blocking for nonspecific binding, the plates were incubated for 1 h at 4°C. Afterwards, the binding buffer was removed and the cells were washed twice with ice-cold phosphate-buffered saline (PBS, pH 7.4). Cell bound radioactivity was subsequently recovered by trypsinization of the wells. Radioactivity in the bound and free fractions was determined with a Wizard gamma-counter (Wallac, Turku, Finland). Specific binding was calculated by subtracting nonspecific from total binding at each concentration of radioligand. Affinity (K_d) and apparent number of binding sites per cell (B_{max}) were calculated from Scatchard plots using Prism software (GraphPad Software Inc.)

Liposomes preparation

The liposomes were prepared by a modified reverse-phase evaporation technique, as previous reported (Accardo et al., 2012). Briefly, the lipid mixture composed of DSPC or DSPC/*Mon*Y-BN-AA1 (1:0.03 molar ratio) was dissolved in a mixture chloroform/methanol (2:1 v/v). The organic solution was added to a 50 mL round-bottom flask, and the solvent was removed under reduced pressure by a rotary evaporator under nitrogen atmosphere. The resulting lipid film was dissolved in 3 mL diethyl ether and the solution was emulsified, by sonication for 30 min in a bath-type sonicator (Branson 3510, Danbury, USA), with 1 mL of citrate buffer at pH 4,0 (150 mM of citric acid; 150 mM of sodium citrate). The resulting emulsion was then placed on the rotary evaporator (Laborota 4010 digital, Heidolph, Schwabach, Germany) and the organic solvent was removed under reduced pressure at 30°C in nitrogen atmosphere. When viscous gel was obtained, the vacuum was broken and the gel was vortexed for about 1 min. Then, the dispersion was placed at rotary evaporator under vacuum for about 15 min. Then, 1 mL of citrate buffer was added and the suspension was heated at 65°C for 30 min. The resulting liposome suspension was then extruded at 65°C, using a thermobarrel extruder system (Northern Lipids Inc., Vancouver, BC, Canada), passing repeatedly the suspension under nitrogen through polycarbonate membrane (Nucleopore Track Membrane 25 mm, Whatman, Brentford, UK) with 0.1 μm pore size. The external buffer was removed by ultracentrifugation (Optima Max E, Beckman Coulter, USA; rotor TLA 120.2) at 80.000 rpm, at 4°C for 30 min, and the liposomes were resuspended with 1 mL of HBS buffer at pH 7.4 (20 mM of HEPES; 150 mM of sodium chloride), giving a suspension with a total lipid concentration 0.010 M. The actual phospholipid concentration of liposomes was determined by the Stewart's assay (Stewart, 1980). Briefly, an aliquot of the liposome suspension was added to a two-phase system, consisting of an aqueous ammonium ferrothiocyanate solution (0.1 N) and chloroform. The concentration of DSPC was obtained by measuring the absorbance at 485 nm into the organic layer.

Radiolabeling of liposomes and *in vitro* binding studies

Radiolabeling and *in vitro* binding assays of the targeted liposomes (DSPC/MonY-BN and DSPC/MonY-BN-AA1), of untargeted liposomes (DSPC/$(C18)_2$DTPA (0.1%)) and of targeted liposomes (DSPC/MonY-BN and DSPC/MonY-BN-AA1) in presence of a 1000 fold excess of cold peptide, at a final concentration of 2×10^{-4} M and incubated for 1 h at 4°C and at 37°C, were performed a previously described (Accardo et al., 2011). Quality control of labeling was obtained by gel filtration on Sephadex G-50 pre-packed columns (Pharmacia Biotech, Piscataway, NJ) (Accardo et al., 2008). Cell bound radioactivity was recovered by trypsinization of the wells. Radioactivity in the bound and free fractions was determined with a Wizard gamma-counter (Wallac, Turku, Finland).

Doxorubicin encapsulation into liposomes

Dox was encapsulated into liposomes by remote loading as already described for the parent liposome formulation DSPC/MonY-BN (Accardo et al., 2011). Briefly, the liposomes suspension were combined with the Doxorubicin at a drug/lipid ratio of 0.2 (w/w), and then incubated at 65°C for 1 h. The amount of encapsulated Dox was determined as follows: 1 mL of liposome dispersion was ultracentrifugated at 80.000 rpm at 4°C for 30 min, the supernatant was carefully removed and Dox concentration was determined by UV/Visible spectrophotometer at 480 nm. The results have been expressed as encapsulation efficiency, calculated as [(TSDox – ASDox)/TSDox] × 100, where TSDox is the theoretical Dox in the supernatant before encapsulation and ASDox is the actual Dox concentration in the supernatant. All liposome preparations were stored at 4°C. Each formulation was prepared in triplicate.

Liposomes characterization

The mean diameter of the liposomes was determined at 20°C by photon correlation spectroscopy (PCS) (N5, Beckman Coulter, Miami, USA). Each sample was diluted in deionizer/filtered (0.22 μm pore size, polycarbonate filters, MF-Millipore, Microglass Heim, Italy) water and analyzed with detector at 90° angle. As measure of the particle size distribution, polydispersity index (P.I.) was used. For each batch, mean diameter and size distribution were the mean of three measures. For each formulation, the mean diameter and P.I. were calculated as the mean of three different batches. The zeta-potential (ζ) of the liposomes surface was measured in water by means of a Zetasizer Nano Z (Malvern, UK). Data of ζ were collected as the average of 20 measurements.

Cytotoxicity assays

Cytotoxicity of DSPC/*Mon*Y-BN-8(3%)/Dox and DSPC/Dox and free Dox was determined as already described

for the parent compound DSPC/MonY-BN/Dox (Accardo et al., 2012). The colorimetric MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay was used. Briefly, 8000 PC-3 cells/well were plated in 96-well plates and incubated overnight to allow cell attachment. The culture medium was removed and cells were incubated with free Dox and liposomes containing 100 and 300 ng/mL Dox. After 8 h the medium was removed and the cells were incubated for an additional 72 h in fresh medium. Plates were then incubated with 20 μL of MTS in 100 μL of culture medium. Finally, 25 μL of 10% SDS were added to all wells to stop of the reaction and mixed thoroughly. The absorbance was measured with a BioRad 680 microliter plate reader (BioRad, BioRad, Corston, UK) at a wavelength of 490 nm. Cells incubated with Dox-free liposomes were used as control. Viability was expressed as percentage of control (mean ± SEM) for each condition. The experiment was performed at least 3 times with triplicate wells. IC_{50} values for each treatment conditions were calculated graphically.

In vivo experiments

Female BALB/c nude mice (6 week old; Harlan Nossan, Italy) were injected subcutaneously (sc.) on the flank with $2–3 \times 10^6$ PC-3 cells, suspended in PBS with 0.1% glucose and mixed with equal volume of Matrigel ™ (BD Biosciences, Bedford), prepared as described above. All procedures were performed within a laminar air-flow cabinet using aseptic technique. Tumor size was measured each second day, starting 28 days following cell inoculation, by the mean of a digital caliper (2 Biological Instruments, Besozzo, VA, Italy) and evaluated as tumor volume (mm^3) calculated by the following formula: long diameter (D) × short diameter $(d)^2/2$. Approximately 5 weeks after cell implantation, when tumors reached an average volume of 170/200 mm^3, mice were randomly distributed into three groups (n = 5) and intravenously (lateral tail vein) administered with the following sterile formulations: saline buffer (control), DSPC/*Mon*Y-BN-AA1/Dox (10 mg/kg), DSPC/*Mon*Y-BN/Dox (10 mg/kg) and DSPC/Dox (10 mg/kg). Drug dosage was adjusted with sterile HBS according to mean body weight for each group. After dosing, mice were also monitored, twice a week, for body weight to assess non-specific drug toxicity. General animal health condition was also evaluated to detect potential side effects, including food and water withdrawal, impaired movement, body weight loss or behavioral changes. Procedures involving animals and their care were in conformity with institutional guidelines that comply with national and international laws and policies. Implanted tumor size was evaluated 1 week following treatments and then every 4 days for the following 16 days. The experiment was ended once control tumors reached a volume close to 1,000 mm^3 and before any sign of deteriorating health. Tumor growth curves, obtained by plotting tumor volume (mm^3) versus time after treatment (days), were generated for the different experimental groups. All animal experiments complied with the Italian D.L. n. 116 of 27 January 1992 and associate guidelines in the European Community Council Directive of 24 November 1986 (86/609/ECC).

Results and discussion

Peptide selection

In order to compare stability and binding properties of a bombesin analog sequence with the [7–14] wild type bombesin peptide, we have synthesized two peptide derivatives with the general formula *DOTA-$(AEEA)_2$-peptide* in which the DOTA chelating agent has been introduced on the N-terminal end of bombesin like peptide and two amino-ethoxy-ethoxy-acetic acid (AEEA) residues have been used to space the active sequence from the DOTA chelating agent. As reported in Figure 1, *DOTA-$(AEEA)_2$-BN* contains the wild type sequence of [7–14]BN, i.e. the same peptide sequence we already used in *Mon*Y-BN, while *DOTA-$(AEEA)_2$-BN-AA1* contains a modified bombesin sequence (BN-AA1) that should yield the desired high serum stability, high receptor affinity and should act as a bombesin antagonist. The peptide sequence (BN-AA1) has been chosen following the indications reported by Maecke et al (Mansi et al., 2009, 2011; Abiraj et al., 2011; Llinares et al., 1999) and by Ametamey et al (Höhne et al., 2008). In the new peptide sequence the Leu^{13}-Met^{14} C-terminal sequence has been replaced by Sta^{13}-Leu^{14} for stabilization against aminopeptidase and N-methyl-glycine has been inserted in place of natural glycine in order to reinforce the Val-Gly bond that could be sensitive to carnitine enzyme *in vivo*. Moreover, as previously reported (Mansi et al., 2009) the replacement of Leu^{13} with the Sta^{13} residue provides antagonist properties to the peptide. Finally, the D-Phe residue on the N-terminal end of the bioactive sequence should increase binding and stability.

Binding and stability studies of peptide derivatives

The *DOTA-$(AEEA)_2$-peptides* were synthesized by Fmoc/tBu chemistry according to standard solid phase peptide synthesis protocols, using Rink-amide MBHA resin as polymeric support (Chang & White, 2000). Both peptide conjugates were collected in good yields after HPLC-RP purification. Analytical liquid chromatography–mass spectrometry (LC-MS) data confirmed the compound identities and their high purity (see Figure 1).

The peptides were labeled with $^{177}LuCl_3$ at specific activities of 31 GBqμmol^{-1} with a labeling yield of >98%. After incubation in human serum at 37°C up to 72 h, the amount of intact peptide was determined using radio-HPLC analysis. The peptide *DOTA-$(AEEA)_2$-BN-AA1* is the most stable with a half-life of 414.1 ± 39.4 h, while the wild-type [7–14]bombesin peptide derivative, *DOTA-$(AEEA)_2$-BN*, show a half-life of only 15.5 ± 2.1 h (Figure 2a). As concerns the binding properties to GRP receptors overexpressed by PC3 cells, the two peptide derivatives labeled with ^{111}In showed a similar

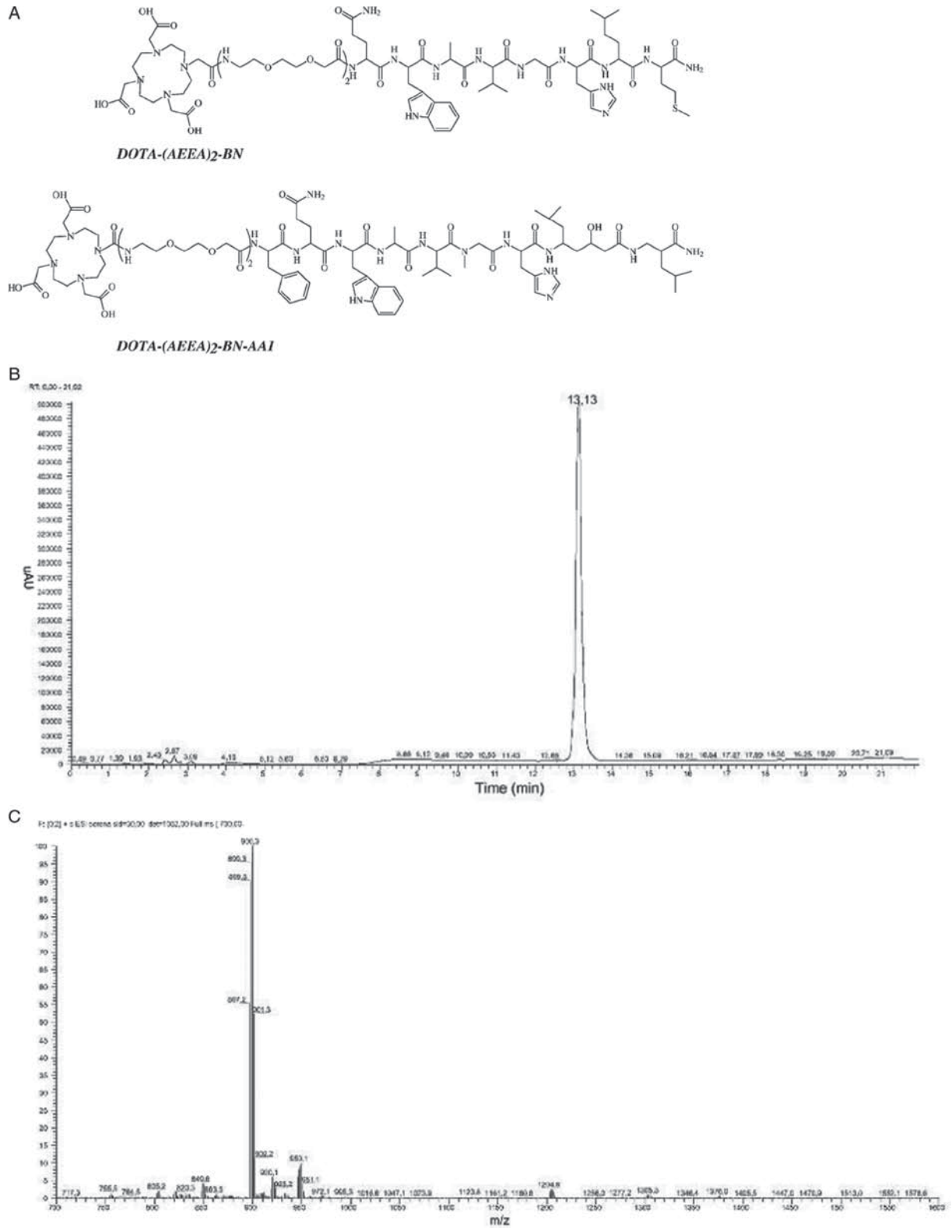

Figure 1. (A) Schematic representation of *DOTA-(AEEA)$_2$-BN* and *DOTA-(AEEA)$_2$-BN-AA1* peptide conjugates; (B) and (C) HPLC chromatogram and ESI mass spectra of *DOTA-(AEEA)$_2$-BN-AA1* peptide, respectively.

behavior with K_d values of 3.30 ± 0.39 nM (mean ± SD) and 5.91 ± 0.57 nM for *DOTA-(AEEA)$_2$-BN* and *DOTA-(AEEA)$_2$-BN-AA1*, respectively; and B_{max} values of $(2.45 \pm 0.07) \cdot 10^5$ and $(3.52 \pm 0.10) \cdot 10^5$ binding sites/cell (Figure 2b).

Liposome formulation and characterization

The new peptide derivative *Mon*Y-BN-AA1 (Figure 3) containing the Bombesin analog peptide BN-AA1, the DTPA chelating agent, the hydrophobic moiety with two C18 alkyl chains, and PEG spacers in a single molecule

was synthesized in order to formulate new liposomes with expected improved properties compared to the DSPC/*Mon*Y-BN liposomes recently developed (Accardo et al., 2012).

The chemical synthesis of *Mon*Y-BN-AA1 was performed in solid-phase by using standard solid-phase-peptide-synthesis (SPPS) procedures based on Fmoc/tBu chemistry using rink-amide resin as polymeric support. The peptide derivative was cleaved from the resin and free peptide precipitated in cold ethyl ether and lyophilized. The crude compound was purified by reversed phase HPLC and characterized by LC–MS analysis and Maldi-TOF.

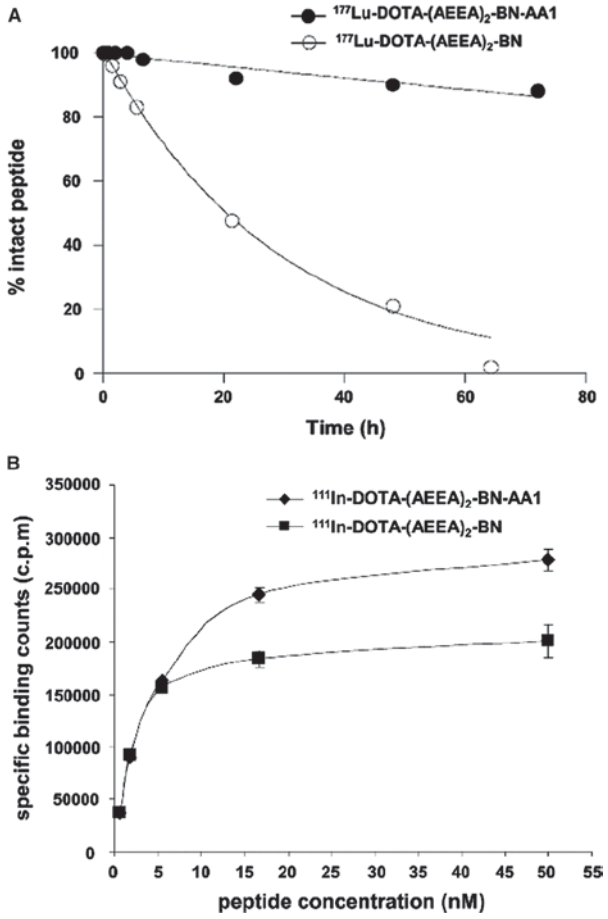

Figure 2. (A) *In vitro* serum stability of *^{177}Lu-DOTA-(AEEA)$_2$-BN* and *^{771}Lu-DOTA-(AEEA)$_2$-BN-AA1* at 37°C; (B) saturation binding curves of *^{111}In-DOTA-(AEEA)$_2$-BN* and *^{111}In-DOTA-(AEEA)$_2$-BN-AA1* on PC-3 cells at 4°C.

Liposomes composed of DSPC/*Mon*Y-BN-AA1 (7.66 mg DSPC, 1.16 mg *Mon*Y-BN-AA1; 97/3 molar ratio) were prepared as already reported for the parent supramolecular aggregate based on DSPC and *MonY*-BN (Accardo et al., 2012), and fully characterized. HBS buffer solution (1.0 mL) was used with a final lipid concentration 0.010 M. The mean diameter, the polydispersity index (P.I.), and zeta potential (ζ) of the mixed liposomes DSPC/*Mon*Y-BN-AA1 (97/3 molar ratio) are 136.3 ± 42.4 nm, 0.20 ± 0.05, and −12.8, respectively, as reported in Table 2. Liposome stability was investigated by DLS measurements: stock solutions of mixed liposomes DSPC/*Mon*Y-BN-AA1 (97/3 molar ratio) at lipid concentration 0.010 M were prepared in pure water and in water buffered at pH 7.4 containing 0.9% w/w NaCl and stored at 4°C and 37°C. Mean diameter and polydispersity index were measured every 7 days. All scattering intensity curves are superimposed indicating that any variation of size and P.I. occur in 1 month.

Doxorubicin was encapsulated in the targeted and untargeted liposomes by the remote loading method. The drug/lipid (w/w) ratio used was of 0.2. The experimental conditions, namely the buffer used in the internal aqueous phase, the incubation time as well as the incubation temperature, have been previously described for the loading of DSPC/*Mon*Y-BN (Accardo et al., 2012). A drug-loading content >90% was achieved preparing liposomes in citrate buffer and incubating them with the drug for 1 h at 65°C. As expected, the loading of doxorubicin in liposomes did not alter morphology and size of the nanovectors (data not shown).

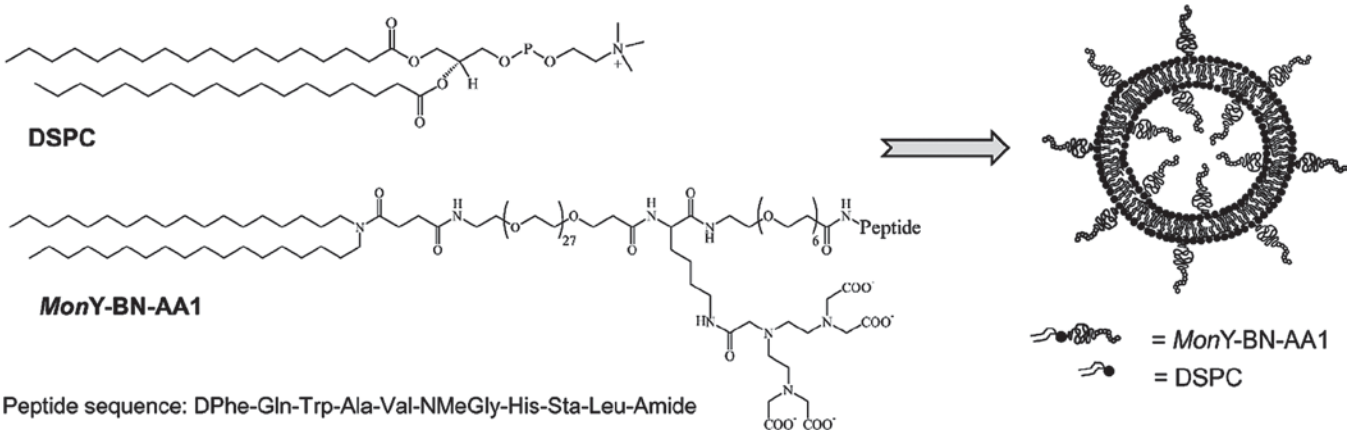

Figure 3. Schematic representation of DSPC phospholipid and *Mon*Y-Peg27(DTPA)-BN-AA1 (*Mon*Y-BN-AA1) amphiphilic molecule and liposomal structure obtained by mixing DSPC/*Mon*Y-BN-AA1 in 97/3 molar ratio. The peptide sequence of bombesin analog is reported using the three-letter amino acid code.

Table 2. Liposome characteristics.

Formulations	Liposome diameter (nm) ± S.D.	P.I. ± S.D.	ζ (mV)
DSPC	161.4 ± 0.10	0.09 ± 0.01	–6.7
DSPC/*Mon*Y-BN-AA1	136.3 ± 42.4	0.20 ± 0.05	–12.8
DSPC/*Mon*Y-BN-AA1/DOX	153.7 ± 35.4	0.13 ± 0.08	–12.0

In vitro studies

Effective binding capability of liposomes decorated with BN and BN-AA1 peptide sequences was confirmed by *in vitro* nuclear medicine studies in PC-3 cell lines at 4°C and 37°C. The labeling of liposomes was achieved by adding trace amounts of $^{111}InCl_3$. Nude DSPC liposomes, containing very low amount of 111-In-DTPA$(C18)_2$ (0.1%), were used as negative control system. A competition assay for BN-AA1 liposomes in presence of an excess of cold peptide was also performed to assess receptor selectivity. Radiolabeling of the liposomes was >95% at 2×10^{-4} M concentration as confirmed by gel filtration. From the inspection of Figure 4a, both mixed formulations containing BN and BN-AA1 peptide sequences (DSPC/MonY-BN and DSPC/MonY-BN-AA1) shown a preferential binding to PC-3 cells (2.7 ± 0.3, 3.0 ± 0.3 at 37°C) with respect to the pure DSPC liposomes (1.4 ± 0.2 at 37°C). Moreover, the competition assay between DSPC/MonY-BN-AA1 and cold peptide (binding data at 0.8 ± 0.3, at 37°C), confirming receptor selectivity. These results confirm that binding activity of both peptide sequences towards cells over-expressing GRP receptors is conserved when the peptides are allocated on the external liposomal surface. As expected, comparable values of binding activity have been found for the wild type sequence of [7–14] BN and for the antagonist sequence.

Cytotoxicity assays of doxorubicin liposomes

The cytotoxic effect of DSPC/*Mon*Y-BN-AA1/Dox liposomes on PC-3 prostate cancer cells was also assessed, and compared to the behavior of the previously studied DSPC/*Mon*Y-BN/Dox liposomes and to the underivatized DSPC/Dox liposomes and to free doxorubicin. PC-3 cells were exposed to liposome formulations (100 μL) containing 500 or 1500 ng of lipids and 100 or 300 ng of Dox, respectively, or to free doxorubicin, for 8 h, and the cell viability was measured by MTS assays (Figure 4). Incubation of cells with both targeted doxorubicin liposomes showed significantly lower cell survival compared to DSPC/Dox treated cells, in the presence of 100 ng/mL and 300 ng/mL drug amounts. The increased potency of targeted liposome with respect to DSPC/Dox can be fully attributed to Dox because control experiments with empty liposome had no significant effect on cell viability (data already reported) (Accardo et al., 2012). The two peptide derivatized liposomal systems, DSPC/*Mon*Y-BN-AA1/Dox and DSPC/*Mon*Y-BN/Dox present a similar cytotoxic activity, as expected by the similar binding properties of the respective labeled DOTA-peptides.

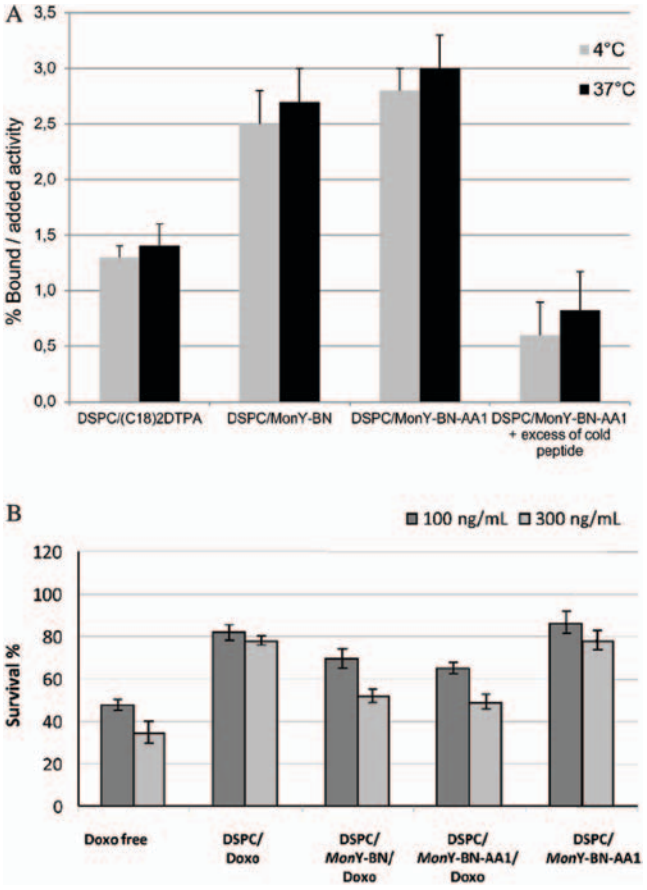

Figure 4. (A) Binding assays of DSPC/*Mon*Y-BN, DSPC/*Mon*Y-BN-AA1 and DSPC/$(C18)_2$DTPA ^{111}In-radiolabeled liposomes on PC-3 cell line overexpressing the GRP at 37°C and 4°C at 1 h; (B) Results of cytotoxicity studies. Human prostatic cancer, PC-3, cells were treated with free Dox or with liposomal Dox compositions: DSPC/*Mon*Y-BN-AA1/Dox, and DSPC/Dox; at 100 or 300 ng of Dox. Control experiments were performed using empty liposomes of DSPC/*Mon*Y-BN-AA1 at the same experimental conditions. Cytotoxicity of DSPC/*Mon*Y-BN/Dox is also reported for comparison. Data are expressed as percent of control. Each value is the mean ± SEM of three experiments performed in triplicate.

In vivo studies

In vivo studies on the therapeutic efficacy of DSPC/*Mon*Y-BN-AA1/Dox targeted liposomes in comparison to already described (Accardo et al., 2012) DSPC/*Mon*Y-BN/Dox targeted liposomes, to DSPC/Dox liposomes and to saline were performed in PC-3 xenograft bearing mice. In mice treated with DSPC/MonY-BN/Dox and DSPC/MonY-BN-AA1/Dox targeted liposome formulations tumor volume was significantly reduced, compared to saline treated mice, at 15 and 19 days post treatment (Figure 5A). Conversely, in mice treated with DSPC/Dox tumor volume was not significantly different from saline group, at any time point considered.

For the new studied formulation, DSPC/*Mon*Y-BN-AA1/Dox, the maximum effect was observed 19 days after treatment (tumor growth inhibition was 43% compared to DSPC/Dox liposomes, and 59% compared to saline group, Figure 5A). It is likely that tumor growth inhibition observed in DSPC/*Mon*Y-BN-AA1/Dox and

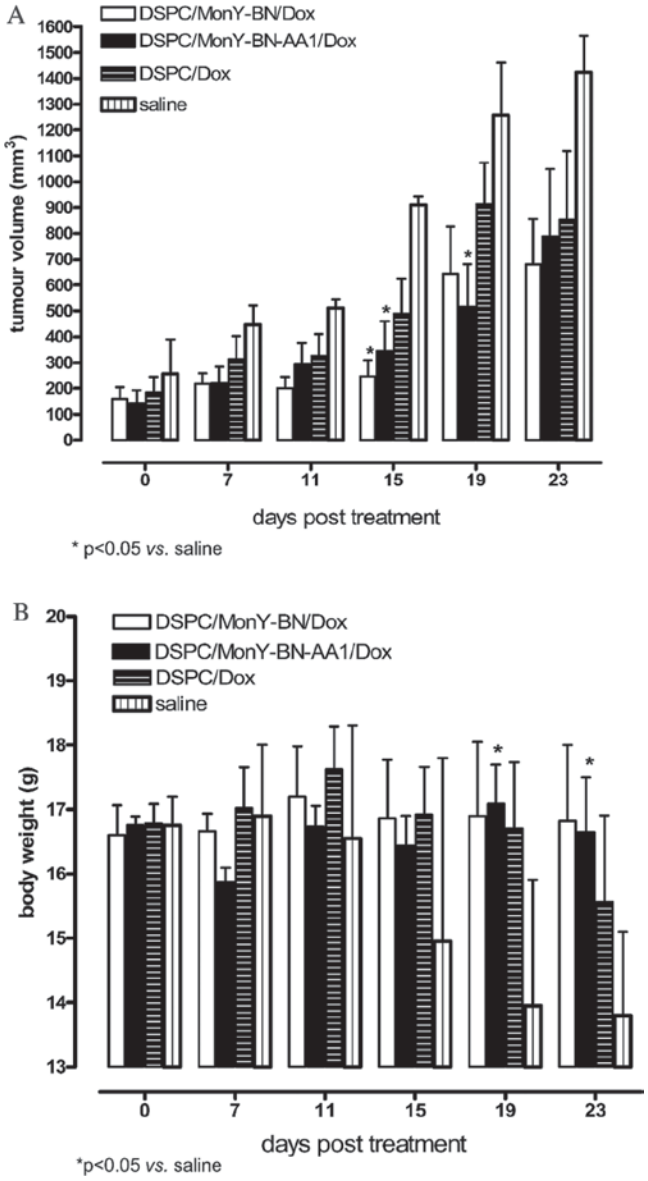

Figure 5. Treatment with targeted and untargeted liposomal doxorubicin DSPC/*Mon*Y-BN-AA1/Dox, and DSPC/Dox ($1.0 \cdot 10^{-2}$ M lipid concentration, 10 mg of Dox/Kg) to evaluate tumor growth in BALB/c nude mice bearing xenografts (PC-3). Treatment with DSPC/*Mon*Y-BN/Dox and with HBS buffer (control) are also reported for comparison. Experiments started approximately 5 weeks after cell implantation. Mice (n = 5) were administered i.v. with injection of liposome suspensions (100 μL) and therapeutic efficacy was assessed by measuring (A) tumor volume (mean ± SD) over time. After 19 days from treatment, DSPC/*Mon*Y-BN-AA1/Dox produces, respect to the saline solution, a TGI of 59%. (B) Mice body weight (g) vs day post-treatment.

DSPC/*Mon*Y-BN/Dox groups, over considered time interval may be due to different kinetics of the targeted liposomes compared to DSPC/Dox group. The hypothesis is that targeted liposomes, by binding to their specific receptors within the tumor, allow to achieve high drug concentrations into the tissue for a longer time than liposomes lacking the targeting ability. This hypothesis needs to be further investigated and confirmed. Body weight was also evaluated for all groups as a measure of therapeutic effect. As shown in Figure 5B, animals treated with both targeted liposomal formulations did not show the same degree of progressive body weight loss as control animals. In these animals, treated with only DSPC/Dox liposomes, the exponential tumor growth observed starting about 10 days following treatment was paralleled by a clear reduction in body weight.

Conclusions

The newly developed DSPC/*Mon*Y-BN-AA1/Dox nanovectors confirm the ability to selectively target and provide therapeutic efficacy in PC-3 cells and PC-3 xenograft bearing mice previously obtained with DSPC/*Mon*Y-BN/Dox. Although the two compounds yield very similar results in the *in vitro* and *in vivo* assays presented, the lack of receptor activation and possible acute biological side effects provided by using the AA1 antagonist sequence should provide safer working conditions for further development of this class of drug delivery vehicles.

Declaration of interest

This work was supported by grants from the Italian Minister of Research (MIUR): Grant FIRB RBRN07BMCT and Grant PRIN E61J11000300001. Accardo, A., Tesauro, D. and Morelli, G., have participation in a spin-off company (Invectors, srl) devoted to the clinical development of the described compounds for cancer therapy. The other authors report no conflicts of interest in this work.

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