Alpha-particle radiotherapy: For large solid tumors diffusion trumps targeting

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running title: Targeted versus non-targeted α -particle therapy for solid tumors

Abstract

Diffusion limitations on the penetration of nanocarriers in solid tumors hamper their therapeutic use when labeled with α -particle emitters. This is mostly due to the α -particles' relatively short range ($\leq 100\mu\text{m}$) resulting in partial tumor irradiation and limited killing.

To utilize the high therapeutic potential of α -particles against solid tumors, we designed *non-targeted, non-internalizing* nanometer-sized tunable carriers (pH-tunable liposomes) that are triggered to release, within the slightly acidic tumor interstitium, highly-diffusive forms of the encapsulated α -particle generator Actinium-225 (^{225}Ac) resulting in more homogeneous distributions of the α -particle emitters, improving uniformity in tumor irradiation and increasing killing efficacies.

On large multicellular spheroids (400 μm -in-diameter), used as surrogates of the avascular areas of solid tumors, interstitially-releasing liposomes resulted in best growth control independent of HER2 expression followed in performance by (a) the HER2-targeting radiolabeled antibody or (b) the non-responsive liposomes. In an orthotopic human HER2-negative mouse model, interstitially-releasing ^{225}Ac -loaded liposomes resulted in the longest overall and median survival.

This study demonstrates the therapeutic potential of a general strategy to bypass the diffusion-limited transport of radionuclide carriers in solid tumors enabling interstitial release from *non-internalizing* nanocarriers of highly-diffusing and deeper tumor-penetrating molecular forms of α -particle emitters, independent of cell-targeting.

keywords: interstitial transport; pH-responsive liposomes; α -particle therapy; Actinium-225; tumor penetration; solid tumors

Introduction

In solid tumors the diffusion-limited penetration depths of radionuclide carriers [1-3] combined with the short range of α -particles (40-100 μm) [4] hamper their use mostly due to partial tumor irradiation. Given the high killing efficacy of α -particles (4-5 tracks across the nucleus result in cell kill) [5-11] which is also mostly independent of cell-oxygenation state unlike β -particles [12] this is quite unfortunate. Carrier molecules that diffuse substantially from the vasculature into the viable tumor have been suggested to be necessary for effective irradiation of the viable solid tumor-cell regions [3].

To address the diffusion-limitations on the transport of radionuclide carriers and the partial tumor irradiation, we designed nanometer-sized lipid carriers (liposomes) that are triggered to release in the tumor interstitium *highly-diffusing forms of encapsulated α -particle emitters*. Given that the molecular-size of the released chelated-emitters is significantly smaller than the nanometer-sized carriers, we expect that the corresponding higher diffusivities may result in more uniform distributions and deeper penetration of radionuclides within tumors, resulting in more even tumor irradiation. Towards this goal, we designed pH-responsive liposomes encapsulating chelated forms of α -particle emitters. These liposomes are expected to demonstrate enhanced uptake by solid tumors as described by the Enhanced Permeability and Retention (EPR) effect [13]. Additionally, in the slightly acidic tumor interstitium ($7.4 > \text{pH} > 6.0$) [14,15] a pH-responsive mechanism on the liposome membrane results in release of these chelated forms of encapsulated radioactivity [16].

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6 The pH-triggered release mechanism is simply based on the formation of *defective*
7 *interfaces* - in terms of lipid packing - *that span the lipid bilayer* resulting in high
8 membrane permeability [16,17]. These lipid interfaces originate at the boundaries of
9 domains formed upon lipid phase separation. Liposome membranes are designed to
10 contain two lipid types: one with a non-titratable headgroup (lipid with gray headgroup
11 in cartoon, Figure 1) and one with a titratable domain-forming headgroup (lipid with red
12 headgroup); the tail lengths are different to enhance lipid packing defects at the interfaces
13 upon phase separation [16,17]. The extent of ionization on the headgroups of the
14 domain-forming lipids is controlled by the pH which is used to shift the balance
15 between electrostatic repulsions and H-bonding attractions [18]. At neutral pH, the
16 headgroups of the domain forming lipids are negatively-charged, opposing close
17 proximity between lipids, resulting in uniform lipid membranes. As the pH is
18 decreased, gradual headgroup protonation minimizes the electrostatic repulsion, and
19 phase separated lipid domains are formed driven mostly by H-bonding [19]. The utility
20 and robustness of this approach has been reported for several agents *in vitro* and *in vivo*
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43 In this study, pH-responsive (interstitially-releasing) liposomes were loaded with the α -
44 particle generator Actinium-225 (^{225}Ac) and were compared to non-pH-responsive
45 (non-releasing) liposomes and to radiolabeled antibodies (Trastuzumab) targeting
46 HER2. We evaluated the distributions of the carriers, and of the delivered radioactivity,
47 in multicellular spheroids comprised of breast cancer cells with variable
48 radiosensitivities and variable expression levels of HER2. Lastly, the efficacy of all
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constructs to prolong survival was evaluated on animals with orthotopic MDA-MB-231 triple negative breast cancer (TNBC) xenograft tumors.

Materials and Methods

Materials

The lipids 1,2-dihexarachidoyl-sn-glycero-3-phosphocholine (21PC), 1,2-distearoyl-sn-glycero-3-phosphate (DSPA), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rhd-lipid) were purchased in chloroform from Avanti Polar Lipids (Alabaster, AL). Poly(2-hydroxyethyl methacrylate) (polyHEMA) was obtained from Polysciences, Inc. (Warrington, PA). All materials are described in detail in the supplemental data. The IgG1 κ isotype control antibody was purchased from SouthernBiotech (Birmingham, AL). Actinium-225 (^{225}Ac , actinium chloride) was provided by the Institute for Transuranium Elements, Germany, as described before [23,24].

Nanocarriers

pH-responsive and non-pH-responsive liposomes were composed of 21PC:DSPA:cholesterol(chol):DSPE-PEG:Rhd-lipid at a mole ratio of 53.7:23.0:9.5:13.0:0.8 and of 21PC:chol:DSPE-PEG:Rhd-lipid at a mole ratio of 66.0:28.0:5.0:1.0, respectively, and were prepared using the thin film hydration method [25]. Lipid suspensions were extruded through 100 nm diameter pores, and were characterized for size and zeta potential using a Zetasizer NanoSeries (Malvern Instruments Ltd.). Liposomes encapsulating 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) were loaded using the A23187 ionophore [26]. Loading efficiency was determined by comparing the Bismuth-213 (^{213}Bi) γ -emissions (360 – 480

keV) at secular equilibrium before and after Sephadex-G-50 separation using a Cobra γ -counter (Packard Instrument Co., Inc.).

To radiolabel antibodies with ^{225}Ac [23,24] or Indium-111 (^{111}In), the isothiocyanate-functionalized metal chelator (DOTA or diethylenetriamine pentaacetic acid (DTPA), respectively) was added in one step as described in supplemental data [27]. Radiochemical purity was assessed via instant thin layer chromatography (iTLC). The ^{111}In -DTPA-Trastuzumab was used to measure the levels of HER2 receptors per cell as reported before [28,29].

Retention of ^{225}Ac was determined by incubating the ^{225}Ac -loaded carriers in HybriCare media supplemented with 10% fetal bovine serum (FBS) at 37°C. At pre-determined time points, aliquots were removed, and released radioactivity was separated either with a Sephadex-G-50 (liposomes) or 10-DG (antibodies) column [26].

The extents of released radioactivity from liposomes corresponding to each of the three possible forms (^{225}Ac -DOTA, ^{225}Ac -ionophore complex, ^{225}Ac) were evaluated by separation and quantitation of two of the forms in addition to the overall radioactivity balance (as described in detail in supplemental data).

Cell and Spheroid Culture

The HER2 positive BT474 cell line, the TNBC MDA-MB-231 cell line, and the HER2-negative MCF-7 cell line which was stably transfected (by Dr. V. Karantzà) to express moderate HER2 levels were obtained from and maintained in ATCC recommended

media supplemented with 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin at 37°C, 5% CO₂. MCF-7/HER2 media was also supplemented with 2 mg/mL G418 antibiotic. The expression of HER2 per cell was evaluated using ¹¹¹In-DTPA-Trastuzumab by measuring the extent of cell-associated antibodies for fixed cell density while varying the antibody-to-cell ratios at 4°C, and by correcting for non-specific binding at 50x excess of non-radiolabeled antibody and for its immunoreactivity, repeated at two different cell densities [29].

Spheroids were initiated by seeding 400 (BT-474) or 250 (MCF-7/HER2) cells/well into polyHEMA-coated 96-well plates with U-shaped wells or by mixing 125 (MDA-MB-231) cells/well with 2.5% w/v MatrigelTM-containing media followed by centrifugation for 5 minutes at 500x gravity, and incubation until spheroids grew to appropriate size (typically at least one week). The number of cells per spheroid were counted following dissociation (by incubation with trypsin) of the corresponding spheroids.

Measurement of Spheroid Interstitial pH-Gradients

The pH gradients of the interstitium of spheroids were determined by incubating - overnight - spheroids with the pH-responsive membrane-impermeant fluorophore SNARF-4F [22,30], and, upon transfer to fresh media, by acquiring optical sections through the spheroid equator using a Leica TCS SP2 confocal laser scanning microscope under a 10x, 0.4 NA dry objective. The ratio of emitted fluorescence intensities at 640 nm over the intensities at 580 nm (ex: 514 nm) - which is a function of pH and is independent of the concentration of the fluorophore [30] - was used to quantitate the pH in the spheroid interstitium using a calibration curve of the fluorophore in media at known pH

values acquired at identical imaging conditions in the absence of spheroids. To evaluate the radial pH gradient of the spheroid interstitium, an eroding algorithm was applied on spheroid images at progressively smaller radii and, within each ring (6 μm thickness), the average measured pixel intensity was calculated and compared to the calibration curve.

Nanocarrier Profiles in Spheroids

Following incubation with FITC-labeled antibodies (see supporting information for antibody labeling) or Rhd-lipid-labeled liposomes, spheroids were either washed and incubated in fresh media (clearance studies), or were immediately placed into optimal cutting temperature (OCT) compound following freezing and cryosectioning into 20 μm -thick slices. Slices were then imaged using transmission fluorescence microscopy by an Olympus IX70 inverted microscope (Olympus America Inc, PA) with a 20x, 0.25 NA dry objective. The radial nanocarrier averaged concentrations were determined using an erosion algorithm averaging within concentric rings of 3 pixels (2.7 μm) width. Calibration curves were generated by measuring the intensities of serial dilutions of known concentrations in a 20 μm -pathlength cuvette.

Actinium-225 Profiles in Spheroids

Spheroids were incubated with ^{225}Ac -labeled carriers for 6 hours, briefly washed (5-10 seconds) by being transferred in fresh media and were then frozen within OCT. Eight- μm -thick spheroid slices were placed on a Zinc-Sulfur-Silver-containing scintillation sheet and were imaged using a digital autoradiography imaging technique (α -Camera) [31]. The α -Camera calibration coefficient y ($\text{Bq}/(\text{pixel intensity unit}\cdot\text{h}^{-1})\cdot 10^{-9}$) for equilibrium ^{225}Ac determined using the method of Chouin et al.[32] is described in detail

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6 in supplemental data. In spheroids, the average activities of ^{225}Ac as a function of radial
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8 position were determined using the eroding algorithm described above (of 11-16 μm
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10 thickness rings).
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14 *Phosphorylation levels of histone γ -H2AX in spheroids*

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16 One hour after completion of the 6 hour incubation with radiolabeled carriers and
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18 subsequent wash, spheroids were fixed, mounted in OCT gel, frozen, and sectioned at 20
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20 μm thickness slices. Following permeabilization, slices were immunostained with an
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22 anti-phospho-H2AX primary antibody (Clone JBW301, 2 $\mu\text{g/mL}$) followed by an
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24 AlexaFluor-555-goat anti-mouse IgG1 according to manufacturer's instructions (see
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26 details in supplemental data). Spheroid slices were imaged using fluorescence
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28 microscopy (Olympus IX81 with a 20x, 0.25 NA dry objective), and an eroding code (as
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30 above) was used to quantitate the radial average distributions of the extent of γH2AX
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32 phosphorylation.
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39 *Cell Monolayer and Spheroid treatment with ^{225}Ac -labeled carriers*

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41 Cells in monolayers or in spheroids were incubated for six hours with cell culture media
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43 containing liposomes (0.1 μmol lipids) or 10 $\mu\text{g/mL}$ Trastuzumab (100x in excess of total
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45 HER2 receptors) at different radioactivity levels, and were then washed with fresh non-
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47 radioactive media. Cell monolayers were evaluated for viability by a colony formation
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49 assay [33] and by the 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
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51 (MTT) assay [26] after two doubling times (one doubling time was 48, 36, and 30 hours
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53 for BT-474, MDA-MB-231 and MCF-7/HER2, respectively). Evaluation of the
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55 radiosensitivity of cells was conducted in suspension following cesium-137 irradiation
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6 and alpha-particle irradiation via a radiolabeled isotype control antibody as described in
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8 supporting information.
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10 Spheroid volume was monitored until the untreated population stopped growing, or until
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12 spheroid disaggregation/cell death. Volume was determined as $V = (1/6) \pi (a b^2)$, where a
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14 and b are the major and minor axes, respectively.
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21 *In Vivo Evaluation*

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23 The maximum tolerated dose (MTD) of all radioactive constructs was determined by
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25 retro-orbitally injecting healthy athymic nude female mice weighing approximately 20
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27 grams with varying doses of radioactivity (3.7, 9.25, 14.8, 18.5, and 33.3 kBq per mouse
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29 or 100, 250, 400, 500, 900 nCi per mouse). MTD was defined as the maximum dose at
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31 which no mouse died or required euthanasia per Institutional Animal Care and Use
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33 Committee protocol (IACUC). For efficacy studies, a small incision was made on each
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35 mouse, allowing injection of 2 million MDA-MB-231 cells suspended in serum-free cell
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37 culture media into the second mammary fat pad on the right side of animals.
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39 Radiotherapy or saline was administered retro-orbitally once tumors reached 100 mm³.
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41 Animal weight, tumor volume, and overall survival were monitored. Per IACUC
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43 protocol, animals were euthanized if they met conditions for euthanasia or if tumors
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45 exceeded 10% of the overall weight of the mouse.
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52 *Statistical analysis*

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Results are reported as the arithmetic mean of n independent measurements $\pm$ the standard deviation. Student's t-test was used to evaluate differences in behavior between constructs. Survival statistics were analyzed using log-rank testing (MedCalc).

Results

Carriers

Tables 1 and 2 show that radiolabeling for all carriers was sufficient and stable. In particular, liposomes were loaded with more than 43.5 ± 12.8 % of introduced radioactivity which was retained at high levels ($>77.6 \pm 6.2$ %) at physiologic conditions (pH = 7.4, 24 hr) in the presence of serum proteins. As expected, only pH-responsive liposomes exhibited significant release of radioactive contents at pH 6.0 (see also Figure S1) corresponding to the mildly acidic pH of the tumor interstium [14,15]. The duration of six hours of incubation in the acidic environment was chosen to represent a conservative estimate of the liposomes' residence time in tumors *in vivo*. The majority (~76%) of released radioactivity from liposomes was measured to be in the form of ^{225}Ac -DOTA chelates followed by approximately 23% in the form of ^{225}Ac -ionophore complexes (Table 1S). The radiolabeling efficiency of antibodies was also above at least 50% of introduced radioactivity (Table 2) which was retained at high levels ($>82.8 \pm 5.9$ %) at physiologic conditions (pH = 7.4, 24 hr) in the presence of serum proteins. The immunoreactivity of the radiolabeled HER2-targeting antibody was also preserved at high levels (86.5 ± 6.2 %, Table 2).

Cell Lines and Spheroids

The HER2 expression by cells harvested from monolayers (Table 3) followed the order: BT474 >> MCF-7/HER2 > MDA-MB-231 (also on Figures S2, S3 and S4). Only on 'large' (400 μm -in-diameter) BT474 spheroids, the expression levels of HER2 by cells was lower than the corresponding cells in monolayers (Figure S5). The interstitial pH gradients of spheroids were found to depend mostly on spheroid size, and pH values at

the spheroid centers were ~ 6.4 - 6.5 and ~ 6.1 - 6.2 for 200 and 400 μm -diameter spheroids, respectively (Figure S6). For BT474, 1266 ± 89 cells were measured per 200 μm -spheroid and 9833 ± 520 cells were measured per 400 μm -spheroid, and for MDA-MB-231, 1093 ± 87 cells were measured per 200 μm -spheroid and 8250 ± 661 cells were measured per 400 μm -spheroid.

Profiles of Carriers, of ^{225}Ac and of induced phosphorylation of histone $\gamma\text{-H2AX}$ in Spheroids

Both liposome types exhibited similar distribution profiles in same-size spheroids independent of HER2 expression levels (Figures 2A and 2B, S7 and S8) and penetrated deeper than the HER2-targeting antibody even in HER2-positive BT474 spheroids. Liposomes did not significantly associate with cells ($< 0.6\%$) (Figure S9). Only liposomes with the interstitially-releasing (pH-releasing) property of encapsulated ^{225}Ac -contents resulted in almost uniform delivery of ^{225}Ac -radioactivity levels (Figure 3, Figure S10) independent of spheroid type. These measurements were conducted on 400 μm -in-diameter spheroids among which only the MDA-MB-231's developed a measurable necrotic core (Figure S11). As expected [34], the spatiotemporal profiles of the HER2-targeting Trastuzumab and of the associated radioactivity depended strongly on HER2-expression levels of cells forming the spheroids (Figures 2A, 2B and, S7 and S9).

In agreement with the distributions of delivered ^{225}Ac -radioactivity, were also the fluorescence distributions attributed to $\gamma\text{-H2AX}$ foci on equatorial spheroid slices shown in Figure 4 (additional images in Figure S12). The intensities from $\gamma\text{-H2AX}$ foci - which

were treated as biomarkers of dsDNA breaks induced by α -particles - were found to be greater close to the spheroid center when radioactivity was delivered by the pH-releasing liposomes, independent of HER2 expression.

Radiosensitivities of Cells and Killing Efficacies in the Absence of Transport Barriers

Colony survival of cells treated with all ^{225}Ac -labeled carriers in the absence of transport-related barriers (Figure S13) demonstrated equal killing, independent of extracellular pH (7.4 vs. 6.5), except, of course, for the cases of specific targeting. The HER2-targeting radiolabeled antibody exhibited greater killing efficacy only on HER2-positive cancer cell lines, in a manner proportional to the cells' corresponding HER2 expression (Figure S13, Table S2). The relative radiosensitivity of cells in suspensions to α -particle (and Cesium-137) irradiation was as follows: MCF-7/HER2 > MDA-MB-231 $\geq$ BT-474 (Figure S14).

Spheroid Growth Control

In the presence of transport barriers, i.e. in the presence of diffusion-limited transport, only on 'small' (200 μm -diameter) HER2-overexpressing BT474 spheroids the targeting radiolabeled antibody resulted in better growth control (Figure 5A). On 'large' (400 μm -diameter) spheroids of BT474 cells the pH-releasing (interstitium-releasing) liposomes *outperformed the targeted* radiolabeled antibody (Figure 5C and Figures S15, S16 for different dose levels). On TNBC MDA-MB-231 spheroids, pH-releasing liposomes exhibited superior growth control independent of spheroid size (Figures 5B and 5D, and Figures S17, S18 for different dose levels) as confirmed by the outgrowth assay (Figure S19). At first sight, this appears to not be corroborated by the spheroid volume change

measurements yet, this is easy to rationalize: due to the presence of MatrigelTM scaffolds - which were necessary to form MDA-MB-231 spheroids - the reported 'decreasing' spheroid volumes for dying cells were not representative. Indeed spheroids treated with pH-releasing liposomes showed the scaffolds becoming empty of cells, and the outgrowth assay on the residual MatrigelTM scaffolds confirmed lack of migrating cells (Figure S19). It is the scaffolding volume that did not shrink and not the spheroid volume. On small MCF-7/HER2 spheroids with moderate levels of HER2, the efficacies of the radiolabeled Trastuzumab and the pH-releasing liposomes were comparable (Figures S20); however, on large spheroids, the pH-releasing liposomes again controlled growth to a greater extent (Figure S21). Non-radiolabeled liposomes did not affect spheroid growth, and non-radiolabeled Trastuzumab affected only the BT474-spheroid growth (Figures S15, S16).

In vivo Efficacy

Although the MTD for all carriers on tumor-free mice was found to be 14.8 kBq per 20-gr-animal (400 nCi per 20-gr-animal), the efficacy studies on mice bearing TNBC orthotopic tumors were performed at 9.25 kBq (250 nCi) per 20-gr-animal because the MTD levels were found to be toxic. In particular, Figure S23_A shows H&E stained sections of the organs of tumor-free mice injected with radioactivity at the MTD (14.8 kBq (400 nCi) per 20 gr animal) at day 82 after administration of radioactivity delivered by liposomes and by the antibody. Histopathology findings included pneumonitis but no noteworthy renal or liver toxicity. Pulmonary inflammation may have been associated with intravascular hemolysis. In addition, there was diminished splenic white pulp. Figure S23_B shows H&E stained sections of the organs of tumor-bearing mice injected with radioactivity at the MTD (14.8 kBq (400 nCi) per 20 gr animal) which died on days

0 and 1 after administration of radioactivity. The histopathology revealed no renal or hepatic toxicities, but appreciable lung inflammation. The latter is possibly associated with increased RBC death. In addition, there was diminished splenic white pulp.

Figure 6 shows that following one administration the pH-releasing liposomes (at 9.25 kBq (250 nCi) per 20-gr-animal) resulted in best (** p -value < 0.05) tumor growth control and longest median and overall survival (36 and 38 days, respectively) relative to both the non-pH-releasing liposomes (24 and 30 days, respectively) and the radiolabeled HER2-targeting antibody (24 and 26 days, respectively), which were indistinguishable from the group that received no treatment. During the efficacy study (at activity levels lower than the MTD), all animals were euthanized because of tumor size per IACUC protocol. Figure S23_C shows H&E stained sections of the organs of tumor-bearing mice injected with radioactivity at 9.5 kBq (250 nCi) per 20 gr animal. Mice were euthanized on days 20 or 30 (Figure 6). No appreciable renal or hepatic toxicity was observed. Indirect signs of intravascular hemolysis was noted but not to the degree observed in mice at higher doses which resulted in death.

Discussion

Alpha-particle emitters are not traditionally considered a viable avenue for the treatment of solid tumors mostly because of the diffusion-limited transport of radionuclide carriers in the tumors resulting in only partial tumor irradiation [3]. In this study we evaluated the therapeutic potential of α -particle emitters on solid tumors using an engineered approach that alleviates the key factor of diffusion-limited transport. For the delivery of α -particle emitters we designed tunable *non-targeting, non-internalizing* liposomes which were triggered within the acidic tumor interstitium to release highly-diffusive forms (due to their smaller size) of their radioactive contents. This approach resulted in more uniform distributions of the delivered radioactivity in solid tumor surrogates (spheroids), and in significantly enhanced radioactivity uptake towards the spheroid center, causing more uniform irradiation of the tumor analogues and better control of spheroid growth. *In vivo*, on an orthotopic xenograft model with low expression of a cell surface molecular marker (HER2), this approach resulted in the longest overall and median survival compared to both liposomal delivery lacking the interstitial release property and to delivery by a HER2-targeting radiolabeled antibody.

Reported therapeutic approaches using charged heavy particles include external beam (for example ^{12}C ions [35,36]) and brachytherapy implants (for example ^{224}Ra -loaded wires emitting α -particles [37,38]). The current study describes a therapeutic approach that is administered systemically, it is expected to reach solid tumors following the EPR effect, and is designed to interstitially release highly diffusing forms of the α -particle emitters as a response to the intrinsic - if it is the case [15,39,40] - mild acidity of tumors.

Our findings on fast and complete liposome clearance from spheroids (Figures S7, S8, S22), combined with the insignificant liposome association with any of the cell types, support our suggestion that the liposomes used in this study are non-targeting and non-internalizing. A similar assumption is proposed for the major form (~74%) of released radioactivity - the ^{225}Ac -DOTA complexes - which did not significantly associate with cells (only 0.9 ± 0.2 % of introduced ^{225}Ac -DOTA was found to associate with cells) and did not appear to penetrate cells at all. However, the remaining ~24% of released radioactivity that was complexed with the A23187 ionophore - which permeates fluid-phase lipid membranes - may have in addition exhibited diffusion across the cells' plasma membranes (12.2 ± 3.8 % of introduced ^{225}Ac -ionophore was found to associate with cells). The latter chemical form of released radioactivity essentially introduces a second type of diffusing radioactive species.

On HER2-overexpressing BT474 spheroids, the interplay between diffusion and cell binding reactivity relative with the actual size for the avascular tumor analogue and with the trajectory length of emitted α -particles was clearly demonstrated. Although for 'small' avascular tumor analogues the radiolabeled antibodies were more effective than the interstitial release approach from liposomes, for 'large' avascular tumor analogues the approach, which relies on improved interstitial diffusion of released radioactivity, was more effective than the targeted radiolabeled antibody (when compared on the same basis of exposure to radioactivity levels and time). It is not clear if this effect would be observed *in vivo*, given the different blood clearance kinetics between liposomes and antibodies; differences in clearance kinetics are expected to affect the corresponding penetration depths and retention times of radionuclide carriers (and/or their radionuclide

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6 contents) within tumors. However, three- and four-fold longer exposure of large HER2-
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8 positive spheroids to the HER2-targeting antibody exhibited non uniform spatial profiles
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10 similar to those described by the binding-site-barrier-effect slightly shifted towards the
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12 spheroid center (Figure S24) [34].
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17 The biodistributions of liposomes in mice have been published before [21] and are
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19 similar to PEGylated liposomes of the same size and overall zeta potential [41].
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21 Liposomes accumulate mostly in the liver and spleen, and have significantly faster blood
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23 clearance times than the antibodies (with elimination half-lives almost 5 times faster than
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25 the elimination half-lives of antibodies) [42]. Radioactive contents of liposomes are
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27 expected to follow the clearance and biodistributions patterns of their carriers which
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29 would include mostly hepatic and splenic uptake for the liposomal contents [21]. The
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31 three forms of encapsulated ^{225}Ac (free ^{225}Ac , ^{225}Ac -DOTA and ^{225}Ac -ionophore), are not
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33 expected to be released from liposomes during their circulation in the blood. The
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35 liposomal contents are triggered to be released in the tumor interstitial space. Free ^{225}Ac
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37 has been reported to accumulate mostly in the liver, spleen and bone [43,44], and the
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39 ^{225}Ac -DOTA is expected to exhibit fast whole body clearance [43].
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46 In this study, at the highest administered radiation doses delivered either by the liposomes
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48 or by the antibodies, common toxicity-related findings were present in the lung and
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50 spleen. Lungs had acute to chronic signs of pulmonary inflammation (pulmonary edema,
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52 type 2 pneumocyte hyperplasia, increased alveolar macrophages) likely impeded gas
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54 exchange. The spleen had increased red pulp hemosiderin accumulation suggestive of
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56 intravascular hemolysis as well as white pulp lymphoid depletion. No studied cases, even
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those at higher radioactivities, had appreciable renal toxicity. In the efficacy study presented in this manuscript, the administered dose (which was below the MTD) did not result in appreciable levels of above described findings.

During blood circulation, the systemically released daughters from the ^{225}Ac decay are not retained by the DOTA chelator neither by these 'small' liposomes [45] nor by the radiolabeled antibodies [46]. Systemic release from carriers of ^{213}Bi , the last of the emitted radioactive daughters, may cause renal toxicities in mice which can be addressed with pharmacologic interventions (e.g. diuretics) [47]. We did not use diuretics in this study. In humans, the bone marrow could be the dose limiting organ upon administration of ^{225}Ac using radiolabeled antibodies [48]. Such liposome studies in humans do not yet exist.

Another potential source of toxicity may arise from the release, back into the blood stream, of a fraction of the highly-diffusing forms of radionuclides from the tumor interstitium. Although these ^{225}Ac -forms are mostly chelated to DOTA, their ^{213}Bi daughters could be toxic to the kidneys, yet they should only correspond to a small fraction of the approximately 2-3% of the injected dose that usually reaches the tumors [49].

The clearance of liposomes from tumors may in principle be delayed by functionalizing the carriers with cancer-cell binding/targeting ligands [50,51]. For the approach presented herein, this functionalization should preferably not result in fast internalization of the nanocarriers by the cancer cells but should enable long binding times of the nanocarriers

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6 on the surface of cells. In this way, longer residence times of the nanocarriers in the
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8 tumor would be enabled possibly resulting in release of greater extents of therapeutic
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10 contents within the tumor interstitium improving efficacy.
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14 The tumor uptake of liposomes is driven by the EPR effect which does not characterize
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16 all of human tumors [52], but should be expected to describe some of the fast growing
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18 tumors in humans [53]. Towards this goal, probe nanoparticles have been suggested to be
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20 used as biomarkers of EPR for patient selection and predictive nanomedicine [54] with
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22 some approaches already in clinical trials [55].
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27 *In vivo* the applicability of this strategy is determined by the extent of acidification of the
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29 tumor interstitium which activates the release process. Such levels of acidity as the ones
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31 studied herein, and even more pronounced ($\text{pH} < 6.5$) [15], have been documented for
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33 patients with different types of tumors including patients with TNBC [39,56,57]. Our
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35 approach of delivering highly-diffusing forms of α -particle emitters *within* the tumors,
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37 obviating the need for targeting, exhibited better efficacy in animals bearing TNBC
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39 xenografts. We suggest, therefore, that our 'diffusion-assisted' approach may ultimately
40
41 offer a therapeutic option to patients with TNBC or with other 'untargetable' cancers for
42
43 which targeted therapies are simply not applicable. Finally, the impact of this approach
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45 may not only be limited to solid tumors which express low levels of known receptors, but
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47 may extend to tumors *with high variability in expression* of known cell surface receptors,
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51 for which targeted approaches exhibit compromised efficacies [58].
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Conclusion

This study introduces a diffusion-assisted approach for the treatment of solid tumors using α -particle emitters. To improve the intratumoral α -particle emitter distributions for more uniform tumor irradiation, this approach relies on fast-diffusing radioactive species which are released within the tumor interstitium from non-targeted liposomes, following general nanoparticle-based delivery rules for tumor uptake.

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Figure 1. pH-triggered release of contents. (left) pH=7.4 during circulation in blood, (right) at tumor interstitial pH. The upper lipid leaflet represents the outer lipid leaflet of liposomes; the lower lipid leaflet represents the inner lipid leaflet of liposomes. Lipid phase separation and domain formation is activated at the tumor interstitial pH ($7.4 > \text{pH} > 6.0$, shown on the right). Black arrows indicate 'leaky' phase boundaries between the different lipid phases.

Figure 2. Carrier distributions. Radial distributions of pH-responsive liposomes (red) and non-pH-responsive liposomes (light red) (labeled with fluorescent lipids), and of the HER2-targeting FITC-labeled antibody (black) within 400 μm-in-diameter spheroids of (A) HER2-positive BT474 cancer cells, and (B) HER2-negative MDA-MB-231 TNBC cells following 6 hour incubation. On the right, characteristic images of the 20 μm-in-thickness equatorial spheroid slices. Errors correspond to standard deviations of n independent spheroids ($n=5$). Spheroid diameters varied up to approximately 10%. Immunoreactivity of the FITC-labeled antibody was: 85.1 ± 7.8 % against HER2-positive BT474 cancer cells.

Figure 3. ^{225}Ac distributions. Radial distributions and characteristic (α -camera) images (of 8 μm -in-thickness equatorial 400- μm -in-diameter spheroid slices) of the ^{225}Ac delivered by pH-responsive liposomes (red), non-pH-responsive liposomes (light red), and of the HER2-targeting radiolabeled antibody (black) within spheroids of (A) HER2-positive BT474 cancer cells, and (B) HER2-negative MDA-MB-231 TNBC cells. Activities were corrected for radioactive decay. Image pixel intensities were corrected for radioactive decay. Spheroids were incubated with 462.5 – 925 kBq/mL (12.5 - 25 $\mu\text{Ci/mL}$) for 6 hours. Errors correspond to standard deviations of n independent spheroids ($n=5$). Spheroid diameters varied up to approximately 10%.

Figure 4. Radial distributions of γ -H2AX foci in spheroid slices treated with immunofluorescent staining for γ -H2AX caused by the delivered radioactivity by pH-responsive liposomes (red), non-pH-responsive liposomes (light red), and the HER2-targeting radiolabeled antibody (black) in 400 μ m-in-diameter spheroids of (A) HER2-positive BT474 cancer cells, and (B) HER2-negative MDA-MB-231 TNBC cells. Errors correspond to standard deviations of n independent spheroids ($n = 6$ for BT-474 and 3 for MDA-MB-231). Spheroids were incubated with 92.5 kBq/mL (2.5 μ Ci/mL) radioactivity for 6 hours. On the right, characteristic images of 20 μ m-in-thickness equatorial spheroid slices. Spheroid diameters varied up to approximately 10%.

Figure 5. Growth control of 'small' 200- μm -in-diameter spheroids of (A) HER2-overexpressing BT474 cells and (B) TNBC MDA-MB-231 cells, and of 'large' 400- μm -in-diameter spheroids of (C) BT474 cells and (D) MDA-MB-231 cells treated for 6 hours with 18.50 kBq/mL (500 nCi/mL) delivered by pH-releasing liposomes (red), non-pH-releasing liposomes (light red), a HER2-targeting radiolabeled antibody (black) and a radiolabeled isotype control antibody (gray). 'No treatment' is shown in white. Growth control in the presence of non-radiolabeled carriers is shown in Figures S15-21. Errors correspond to standard deviations of repeated measurements ($n = 3$ independent liposome or antibody preparations, 5-8 spheroids per type of carrier per preparation). On the right, characteristic bright field images of spheroids. Spheroid diameters at time of treatment varied up to approximately 10%. * p -values < 0.01, ** p -values < 0.05.

Figure 6. (A) animal survival and (B) growth of orthotopic HER2-negative MDA-MB-231 tumors following one i.v. administration when tumors reached 100 mm³ of 9.25 kBq (250 nCi) of ²²⁵Ac-loaded pH-responsive liposomes (red, *n*=8), ²²⁵Ac-loaded non-pH-responsive liposomes (light red, *n*=8), and of the HER2-targeting radiolabeled antibody Trastuzumab (black, *n*=8). Animal response to non-radiolabeled liposomes was indistinguishable from no-treatment (saline; white symbols, *n*=6) [59]. Error bars correspond to standard errors of the mean. ***p* < 0.05 using the Kaplan–Meier method (log-rank test); **p* < 0.01 by Student's t-test for tumor volumes.

Table 1

Characterization of pH-responsive and non-pH-responsive liposomes. Errors correspond to standard deviations of n independent liposome preparations.

Nanocarrier type	Size, nm (PDI) (n)	Zeta potential, mV (n)	% Loading efficiency (n)	Radioactivity retention in serum after 24 hours at pH = 7.4 (n)	Radioactivity retention in serum after 6 hours at pH = 6.0 (n)
pH-responsive liposomes	114 ± 8 (0.09 ± 0.03) ($n = 6$)	-6.8 ± 2.3 ($n = 6$)	43.5 ± 12.8 ($n = 6$)	77.6 ± 6.2 ($n = 6$)	48.6 ± 5.8 ($n = 6$)
non-pH-responsive liposomes	108 ± 15 (0.12 ± 0.04) ($n = 6$)	-0.8 ± 2.8 ($n = 6$)	61.0 ± 10.1 ($n = 6$)	81.8 ± 4.5 ($n = 6$)	78.8 ± 4.6 ($n = 6$)

Table 2

Characterization of radiolabeled antibodies. Errors correspond to standard deviations of n independent preparations.

Nanocarrier type	% Loading efficiency (n)	Radiochemical purity % (n)	% Immunoreactivity (n)	Radioactivity retention in serum after 24 hours at pH = 7.4 (n)
HER2-targeting Ab	70.1 ± 17.6 ($n = 6$)	95.6 ± 1.9 ($n = 6$)	86.5 ± 6.2 ($n = 6$)	88.2 ± 4.4 ($n = 5$)
Isotype-control Ab	54.1 ± 21.0 ($n = 4$)	97.2 ± 1.1 ($n = 4$)	1.0 ± 0.8 ($n = 4$)	82.8 ± 5.9 ($n = 4$)

Table 3

Measured expression of HER2 copies per cell using cells harvested from monolayers.

Errors correspond to standard deviations of n independent measurements.

Cell Line	HER2 copies per cell (n)
BT474	$1.3 \pm 0.1 \times 10^6$ ($n=5$)
MCF-7/HER2	$1.6 \pm 0.4 \times 10^5$ ($n=2$)
MDA-MB-231	$8.3 \pm 0.8 \times 10^4$ ($n=3$)