Targeted and intracellular triggered delivery of therapeutics to cancer cells and the tumor microenvironment: impact on the treatment of breast cancer

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Abstract  Limiting tumor invasion to the surrounding healthy tissues has proven to be clinically relevant for anticancer treatment options. We have demonstrated that, within a solid tumor, it is possible to achieve such a goal with the same nanoparticle by intracellular and triggered targeted drug delivery to more than one cell population. We have identified the nucleolin receptor in endothelial and cancer cells in tissue samples from breast cancer patients, which enabled the design of a F3-peptide-targeted sterically stabilized pH-sensitive liposome. The clinical potential of such strategy was demonstrated by the successful specific cellular association by breast cancer cells harvested from tumors of patients submitted to mastectomy. In vitro, the nanoparticle targeted the nucleolin receptor on a cell and ligand-specific manner and improved cytotoxicity of doxorubicin (used as a model drug) towards breast cancer and endothelial cells by 177- and 162-fold, respectively, relative to the commercially available non-targeted non-pH-sensitive liposomes. Moreover, active accumulation of F3-targeted pH-sensitive liposomes into human orthotopic tumors, implanted in the mammary fat pad of nude mice, was registered for a time point as short as 4 h, reaching 48% of the injected dose/g of tissue. Twenty-four hours post-injection the accumulation of the dual-targeted pH-sensitive nanoparticle in the tumor tissue was 33-fold higher than the non-targeted non-pH-sensitive counterpart. In mice treated with the developed targeted nanoparticle significant decrease of the tumor viable rim area and microvascular density, as well as limited invasion to surrounding healthy tissues were observed (as opposed to other tested controls), which may increase the probability of tumors falling in the category of “negative margins” with reduced risk of relapse.

Keywords Drug targeting · Triggered drug release · Angiogenesis · Tumor microenvironment · Breast cancer

Introduction

Breast cancer arises in the twenty-first century as one of the leading causes for mortality in women in the western civilization [1]. Nevertheless, adverse side effects and eventual failure of conventional therapy, owing to drug resistance and metastatic disease, are not inevitable outcomes of anticancer treatments.

Tumors are live entities and their survival depends on the immune system [2, 3], surrounding cells [4],
orchestrated pathways [5], and processes [6]. Choosing more than one target from the pool of tumor-stroma interactions, such as the angiogenic blood vessel network, which ensures tumor survival, growth and metastases [7], can profoundly benefit therapeutic approaches. Blood vessels are excellent targets, since they are readily accessible to intravenously administered therapy, avoiding problems related with poor drug penetration into the tumor owing to high interstitial pressure gradients [8]. Moreover, treatment selectivity against proliferative tumor-derived endothelial cells is likely to be achieved, because angiogenic blood vessels overexpress specific molecular markers at their surface (which distinguishes them from the vasculature of individual organs). Inhibition of the angiogenic process and neovasculature normalization are also beneficial for anticancer therapies, as they decrease hydrostatic and interstitial fluid pressure, favoring permeation from the blood stream into the tumor interstitial space, where therapeutic agents can accumulate and be specifically internalized by tumor cells [9].

The prevailing new rationale aims at the development of targeted selective therapies to the tumor microenvironment on the basis of characterized mechanisms, with the possibility of directing and concentrating a therapeutic agent only at the desired target site, while improving access to intracellular sites of action. If the same targeted system is capable of identifying a common target and perform its therapeutic action in the selected cell populations, improved clinical outcomes are expected. The nucleolin receptor is one such target, since it is overexpressed on the surface of cancer and endothelial cells of tumor blood vessels (as well as in the cell nucleolus and cytoplasm) [10, 11], providing the opportunity to develop multi-targeting strategies toward the tumor microenvironment. A possible ligand for this receptor is the F3 peptide (KDEPQRKARLPSQRSR) [16] (Fig. 2b), which binds to the NH2-terminal domain of nucleolin [12].

This study aimed at assessing the therapeutic impact of targeting two different populations within the tumor microenvironment (cancer and endothelial cells from angiogenic blood vessels), using the same F3-targeted poly(ethylene glycol) (PEG) sterically stabilized pH-sensitive liposomes containing doxorubicin (DXR), used as a model drug. It is expected that this formulation, composed of lipids that form a stable lipid bilayer at neutral or basic pH, but destabilize in an acidifying endosome, following receptor-mediated endocytosis [13, 14], may significantly increase the intracellular drug concentration, further facilitating the access of the encapsulated drug to its intracellular site of action.

To our knowledge, specific intracellular triggered release of a payload to two distinct populations of tumor cells was achieved for the first time with the same technological platform, which differentiates it from those commercially available and reported in the literature [15].

Results

Ex vivo cellular association

The nucleolin receptor was identified in cancer cells and endothelial cells from angiogenic blood vessels in the tumor mass of 30 tissue samples obtained from patients diagnosed with invasive breast carcinoma, but no positive staining was depicted in mature blood vessels and mammary ducts in the mammary parenchyma (Fig. 1A). Patients with ages between 45 and 89 years old had not been submitted to chemotherapy or radiotherapy prior to mastectomy. Cells were harvested from each tumor after mastectomy and incubated with F3-targeted and non-targeted liposomes, labeled with rhodamine. In 23 cases, the different cellular levels of rhodamine, quantified by flow cytometry, clearly demonstrated the ability of human breast cancer cells to bind and internalize the targeted formulation (P < 0.001) (Fig. 1B). No correlation was found between the extent of cellular internalization and age, estrogen, progesterone and HER2 receptor expression, nor with proliferation status. These results were obtained from institutional pathology reports and revised by experienced pathologists from the Portuguese Institute of Oncology, Coimbra. The extent of uptake of F3-targeted liposomes by cancer cells was only correlated with the expression of the nucleolin receptor.

In vitro cellular association studies

Cellular association was studied in vitro on nucleolin receptor-overexpressing cancer cell lines (MDA-MB-435S, MDA-MB-231 and Hs578T) and those with low levels of expression (T47D and MCF-7). The 5.1-fold difference on the cellular association of F3-targeted liposomes at 37°C, between the first and second groups (Fig. 2a) clearly emphasized the ability of the F3 peptide to target liposomes on a cell-dependent manner. Within each of the three cell lines overexpressing the target receptor, cellular association differences within the range of 9.7- to 17-fold between F3-targeted liposomes and the non-targeted counterpart, or 2- to 10.4-fold in comparison with liposomes targeted by a non-specific peptide (ARALPSQRSR) [16] (Fig. 2b), indicated that the interaction between F3-targeted liposomes and the tested target tumor cells was also ligand specific. Results on the uptake at 4°C, a temperature not permissive to endocytosis, indicated that F3-targeted liposomes were being internalized, in an energy-dependent manner.
This ligand-specific interaction and cell internalization was further confirmed by confocal microscopy with the cancer cell line (MDA-MB-435S) that had shown the highest levels of uptake of targeted liposomes and with an endothelial cell line of angiogenic blood vessels (HMEC-1) [17], overexpressing the nucleolin receptor [18], thus also reinforcing the dual cell target ability of the developed nanoparticle (Fig. 2c). Calcein was used as a marker for the liposome’s content, and loaded in self-quenched concentrations. Pronounced diffuse intracellular green staining, observed after incubation with the F3-targeted pH-sensitive nanoparticle, evidenced a more effective intracellular delivery of the payload in both cancer and endothelial cells from angiogenic vessels, in contrast with F3-targeted non-pH-sensitive liposomes.

Mechanistic studies aiming at investigating the cell entry pathway of the targeted liposomes, on pre-incubation with drugs that selectively compromised different pathways (Supplementary Fig. 1), and competitive inhibition studies as well (Supplementary Fig. 2), were further performed on cancer (MDA-MB-435S) and angiogenic endothelial (HMEC-1) cell lines. These studies indicated that F3-targeted liposomes were internalized by a receptor-mediated mechanism, most likely through the clathrin-mediated endocytic pathway.

Taken together, these results evidenced that specific internalization by the target cells through the endocytic pathway, followed by inclusion in early endosomes, which mature into late endosomes (with decreased pH) [13], allowed F3-targeted pH-sensitive liposomes to undergo destabilization and consequently release their content in a more effective manner than the non-pH-sensitive counterpart, as demonstrated by the more pronounced staining with calcein in cellular association studies assessed by confocal microscopy.

Cytotoxicity studies

Coupling of the F3 peptide to pH-sensitive liposomes containing DXR resulted in neutral-targeted nanoparticles adequate for systemic administration, with a mean size of 170 ± 12 nm, encapsulation efficiency close to 100% and loading of 150 ± 20 µg DXR/µmol phospholipid. The cytotoxicity of DXR, either free or encapsulated in F3-targeted or non-targeted (non-pH-sensitive or pH-sensitive) pegylated liposomes, was compared as a function of incubation time. IC₅₀ values decreased as the exposure of cells to the drug increased from 1 to 48 h (Table 1). F3-targeted pH-sensitive liposomes were more effective at promoting cancer and endothelial cell death than any other tested formulations, for the reason of their unique feature of combining dual cell targeting and intracellular triggered release of the payload. At 24 h, targeted
pH-sensitive liposomes were 10- and 37-fold more cytotoxic than the targeted non-pH-sensitive formulation against MDA-MB-435S and HMEC-1 cells, respectively. When compared to the commercially available non-targeted non-pH-sensitive liposomes, they were 177- and 162-fold (P < 0.001) more cytotoxic against MDA-MB-435S and HMEC-1 cells, whereas targeted non-pH-sensitive liposomes were only 17- and 4.4-fold more effective, respectively.

These results, reinforced by the previous confocal microscopy observations and by the non-cytotoxic nature of empty liposomes (data not shown), indicated that
binding and internalization and triggered release of targeted liposomes are key aspects for delivery strategies, contributing to a dramatic improvement on cytotoxicity.

Free DXR had the highest levels of cytotoxicity in vitro, but this study, like any in vitro cytotoxicity experiment involving free DXR, does not take into account the widely known unfavorable pharmacokinetics and biodistribution presented by the free drug in vivo [19].

Impact on the angiogenic process

HMEC-1 cells, cultured in serum-free GFR-Matrigel, were organized in capillary-like structures. Following incubation with DXR-loaded liposomes (non-targeted, F3-targeted or targeted by a non-specific peptide), immediately after plating, a decreased and disorganized assembly was evident, particularly with the F3-targeted formulation.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Cytotoxicity of several formulations of DXR against MDA-MB-435S or HMEC-1 cell lines</th>
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<tbody>
<tr>
<td>IC₅₀(µM) ± SD</td>
<td>IC₅₀(µM) ± SD</td>
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<tr>
<td>HMEC-1</td>
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<td>Time (h)</td>
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<tr>
<td>1</td>
<td>0.434 ± 0.030</td>
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<tr>
<td>3</td>
<td>0.401 ± 0.059</td>
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<tr>
<td>24</td>
<td>0.072 ± 0.010</td>
</tr>
<tr>
<td>48</td>
<td>0.058 ± 0.008</td>
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<tr>
<td>MDA-MB-435S</td>
<td></td>
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<tr>
<td>Time (h)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.608 ± 0.460</td>
</tr>
<tr>
<td>3</td>
<td>1.449 ± 0.270</td>
</tr>
<tr>
<td>24</td>
<td>0.232 ± 0.045</td>
</tr>
<tr>
<td>48</td>
<td>0.113 ± 0.078</td>
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</table>

DXR-containing F3-targeted pH-sensitive (pSLF3 [DXR]) liposomes were more effective at causing cancer (MDA-MB-435S) and endothelial (HMEC-1) cell death than F3-targeted non-pH-sensitive (SLF3 [DXR]) or non-targeted, either non-pH-sensitive (SL [DXR]) or pH-sensitive (SL [DXR]) liposomes, for 1, 3, 24, and 48 h exposure to DXR. Data are means ± SD of three independent experiments, each done in triplicate.

Fig. 3 Effect of DXR-containing liposomes on (a, b) vessel formation and (c) vessel destabilization. HMEC-1 cells were incubated with either pH-sensitive (p) or non-pH-sensitive targeted (SLF3), non-targeted (SL) or liposomes targeted by a non-specific peptide (SLNS), on GFR-Matrigel coated plates. Results on b are expressed as percentage of blood vessel reduction normalized to the untreated control. Results on c are expressed as percentage of vessel destabilization as described in “Methods” section. Asterisk symbols represent the significance level of the difference between formulations (two-way ANOVA: **P < 0.01; ***P < 0.001)
Vessel formation was reduced in 71 and 82.1% after an overnight incubation of HMEC-1 cells with F3-targeted DXR-containing liposomes (pH or non-pH-sensitive, respectively), whereas with non-targeted, pH-sensitive or liposomes targeted by a non-specific peptide, vessel formation was reduced in 37.2 and 60%, respectively (Fig. 3b).

The impact on the disruption of capillary-like structures was evident at 16 h. Even though no significant differences were seen between the targeted formulations, the highest level of significance to the non-targeted non-pH-sensitive control was registered with F3-targeted pH-sensitive liposomes \((P < 0.001)\) (Fig. 3c), likely owing to the more efficient intracellular delivery of DXR to the target cells. These results evidenced the anti-angiogenic potential of the targeted triggered release formulation on vessel formation and disruption.

Assessment of in vivo performance

The choice of a mammary fat pad implanted tumor model for in vivo experiments was by reason that an orthotopic location can better mimic the microenvironment’s interference in cancer development, and that the selected breast cancer cell line for tumor induction, MDA-MB-435S, was originally collected from the pleural effusion of a female breast cancer patient [20, 21].

Biodistribution studies

Mice were injected in the tail vein with radiolabeled \(^{3}H\)CHE liposomes containing \(^{14}C\)DXR, non-targeted or F3-targeted, either non-pH-sensitive or pH-sensitive.

Stable drug retention during circulation was observed until 24 h after injection of liposomes. Drug-to-lipid ratio was close to 1 in plasma, suggesting that neither the targeting ligand nor the lipid composition, namely of the pH-sensitive formulation, affected the nanoparticles’ stability.

F3-targeted liposomes were more prone to specifically accumulate in the tumor and deliver DXR to the target site, for a time point as short as 4 h after systemic injection. Twenty-four hours after injection, tumor accumulation of the \(^{3}H\)CHE label from F3-targeted pH-sensitive liposomes surpassed the values obtained with the other tested formulations: accumulation was approximately 2-fold higher than the targeted non-pH-sensitive liposomes, and 57- and 33-fold higher than the non-targeted pH-sensitive or non-pH-sensitive counterparts, respectively. At this time point, significant differences in DXR accumulation in the tumor were only registered between the targeted pH-sensitive formulation and the non-targeted non-pH-sensitive control \((P < 0.05)\) (Fig. 4).

Moreover, 4 and 24 h post-injection, targeted pH-sensitive liposomes were the only ones reporting significant differences of accumulation in the spleen and liver \((P < 0.001)\) to the non-targeted non-pH-sensitive formulation. The extent of DXR accumulation of the former was also significantly lower in the liver \((P < 0.01)\) and spleen \((P < 0.001)\), in comparison to the targeted non-pH-sensitive counterpart. The non-targeted formulations were preferentially cleared from the blood stream by the spleen, owing likely to the absence of specific tumor targeting.

In compliance to these data was the DXR tumor-to-heart ratio of 80.8 for F3-targeted pH-sensitive liposomes versus 3.3 and 12.2 for the non-targeted and targeted non-pH-sensitive liposomes, respectively, 24 h post-injection. This is suggestive of a decrease on the potential dose-cumulative toxicity to the heart, namely relative to the commercially available non-targeted formulation (data not shown).

Results also revealed that in the murine tumor model used, none of the nanoparticles were able to cross the blood–brain barrier.

Therapeutic studies

Aiming at assessing the importance of an established tumor vessel network on the treatment efficiency of F3-targeted nanoparticles, treatment of Balb/c nude female mice bearing orthotopically implanted MDA-MB-435S tumors started 25 days after inoculation of cancer cells. Five groups of eight mice/group were injected in the tail vein with free DXR or DXR-containing non-targeted or F3-targeted non-pH-sensitive or pH-sensitive liposomes. One group of untreated mice receiving saline injections was also used as control.

F3-targeted pH-sensitive liposomes induced significant reduction in the viable rim area as compared to other control treatments: non-targeted non-pH-sensitive \((P < 0.05)\), free DXR \((P < 0.001)\), and saline \((P < 0.001)\) (Fig. 5a). That formulation led to a more evident cell death in the periphery of the tumor (Fig. 5b) and to a stronger effect on tumor necrosis than any other tested treatment. This effect was consistent with the reduction of vessel density (Fig. 5c), suggesting that tumors were more susceptible to treatment with the targeted and triggered drug release formulation.

Despite the absence of significant differences between both targeted formulations, in respect to the extent of the viable rim area and mean vascular density, tumors treated with targeted pH-sensitive liposomes presented a more extensive necrotic area, mainly at the periphery of the tumor (Fig. 5b), and did not evidence whatsoever signs of invasion to the adjacent breast parenchyma and muscle tissue, as opposed to all other tested formulations (Fig. 6). This is a clear evidence of the therapeutic potential of the
developed targeted pH-sensitive formulation in limiting tumor progression to healthy tissues.

Discussion

Currently, sole anticancer approaches do not seem sufficient to impair tumor progression, owing to the elevated interstitial pressure (which hinders drug penetration into the tumor mass), and to the presence of a viable rim surrounding the central necrotic area, where cells continue to grow with the support of the adjacent normal and functional tissues [22]. Angiogenesis actively contributes to tumor growth and its inhibition can be an important means to impair tumor progression and to improve therapeutic access to cancer cells.

We were able to demonstrate the benefits of targeting more than one cell population in the tumor microenvironment with the same nanoparticle, taking advantage of the overexpression of nucleolin, a cellular receptor identified in the membrane of both endothelial and cancer cells [23]. We conducted an analysis of 30 tissue samples obtained from patients with breast cancer. Nucleolin was positively identified in 77% of cases, which supported the development of a nanoparticle for the specific delivery of a cytostatic and antiangiogenic drug to cancer and endothelial cells. The developed F3-targeted lipid-based nanoparticle was endowed with the ability to release intracellularly its payload on a specific triggered release mechanism, based on the pH decrease throughout the endocytotic pathway. This is the first time, according to the authors’ knowledge, that a pH-sensitive nanoparticle combines stability in the bloodstream, increased retention of the encapsulated drug and dual targeting to a solid tumor, with proven tumor accumulation and therapeutic efficiency. Others had previously used pH-sensitive liposomes (with a lipid composition different from the one used herein) to target cancer cells from hematologic tumors, which are more easily accessed than in a solid tumor.

Fig. 4 Biodistribution of radiolabeled F3-targeted and non-targeted liposomes loaded with DXR. Double-labeled ([3H]CHE and [14C]DXR) F3-targeted pH-sensitive liposomes (pSLF3, black bars) accumulated preferentially in the tumor as compared to the liver and spleen, for a time point as short as 4 h and until 24 h after systemic injection, when compared to F3-targeted non-pH-sensitive (SLF3, patterned bars) or non-targeted liposomes, either non-pH-sensitive (SL, clear bars) or pH-sensitive (pSL, gray bars). Each bar represents the average of three mice (±SD). Asterisk symbols represent the significance level of the difference between the referenced formulations and non-targeted non-pH-sensitive liposomes (two-way ANOVA: *P < 0.05; **P < 0.01; ***P < 0.001)
Fig. 5 Treatment of tumor-bearing mice with DXR-containing liposomes. Twenty-five days after MDA-MB-435S tumor cells inoculation in the mammary fat pad, mice were treated intravenously, every week for 5 weeks, with 5 mg DXR/kg either free or encapsulated in F3-targeted pH-sensitive (pSLF3) liposomes, non-targeted non-pH-sensitive (SL) or pH-sensitive (pSL), liposomes targeted by a non-specific peptide non-pH-sensitive (SLNS) or pH-sensitive (pSLNS), and F3-targeted non-pH-sensitive (SLF3) liposomes. One group of non-treated mice injected with HBS was also included. a Viable rim area, b cell death at the periphery (original magnification ×10), and c mean vascular density (MVD) index were determined. F3-targeted pH-sensitive liposomes (pSLF3) evidenced a significant reduction in the tumor viable rim area with evident signs of extensive cell death at the periphery (red arrow) and decreased intratumoral MVD index, as compared with mice receiving DXR, either free or encapsulated in non-targeted non-pH-sensitive liposomes, or with non-treated mice. The most important comparisons between the treatment groups are evidenced in both graphs. Each bar represents the average values within each group (±SD). Asterisk symbols represent the significance level of the difference between formulations (one-way ANOVA: *P < 0.05; **P < 0.01; ***P < 0.001).

Fig. 6 Tumor cell invasion to the surrounding healthy tissues. Malignant cells of tumor-bearing mice treated with DXR-containing F3-targeted pH-sensitive liposomes (pSLF3) did not invade the surrounding tissues, being constrained by a fibrotic pseudocapsule. In opposition, invasion of tumor cells (red arrows) through the pseudocapsule to muscle fibers and mammary parenchyma located near the tumor was observed in groups treated with DXR-containing non-targeted non-pH-sensitive (SL), targeted non-pH-sensitive (SLF3) or non-targeted pH-sensitive liposomes (pSL), as well as with free DXR and in non-treated mice (HBS) (original magnification ×20).
The targeted triggered release formulation resulted in a modest increase in therapeutic efficacy in vivo, in comparison with non-pH-sensitive counterpart, due to a faster drug release and blood clearance.

Cellular association studies in cancer cells harvested from tumors of patients diagnosed with breast cancer demonstrated that liposomes targeted to the nucleolin receptor by the F3 peptide were specifically internalized, owing to the overexpression of the nucleolin receptor on the surface of target cells. This is clinically relevant when considering other receptors in breast tumors, such as the HER2, associated with increased cell proliferation, anti-apoptotic signals, and invasion through activation of tumor-associated proteases [26, 27]. Amplification of the HER2/neu gene or overexpression of its protein product is only observed in 20–30% of human breast cancers and, therefore most patients are upfront ineligible for the treatment [28]. In the 30 case study herein presented, specific cellular association of F3-targeted liposomes was depicted in HER2 positive and negative cells, both indicating positive nucleolin receptor expression. This foresees promising outcomes for patients with developed resistance to anti-HER2 therapy regimens, such as trastuzumab (Herceptin®), and can represent a breakthrough on the treatment of breast cancer, regardless of HER2 eligibility.

F3-targeting also altered the biodistribution profile of pegylated liposomes containing DXR, with a favorable accumulation in the tumor area of a MDA-MB-435S-derived orthotopic murine model of cancer. The remarkable increase in F3-targeted pH-sensitive liposomal tumor uptake of 48% of the injected dose, for a time point as short as 4 h, was likely a consequence of the unique property of the targeting ligand to selectively bind to endothelial cells of tumor blood vessels that are promptly accessible on systemic injection. The control non-targeted non-pH-sensitive formulation was preferentially cleared from the blood stream by the spleen and liver, owing to lack of targeting specificity. These findings might be a breakthrough in drug delivery to solid tumors. In the work of Moreira et al., using a sole tumor cell targeting approach, accumulation of antagonist-G-targeted liposomes in a subcutaneous tumor model of small cell lung cancer ranged between 4 and 5% of the injected dose/g of tissue and only detected 48 and 72 h after systemic administration [29, 30]. In the work of Ishida et al., only 9–13% of the injected pH-sensitive liposomes remained in the blood at 8 h after systemic administration [31].

Preferential accumulation of targeted pH-sensitive liposomes in the tumor tissue resulted in a decreased viable rim area, reduction of mean vascular density and increased necrosis in the central and peripheral areas of the tumor. The group treated with DXR-containing F3-targeted pH-sensitive liposomes was the only one presenting a reduced viable rim area significantly different from the non-targeted non-pH-sensitive treated group (P < 0.05), and from the group treated with free DXR (P < 0.001). It presented extensive cell death at the periphery, where it would be expected to observe tumor proliferation by reason of proximity to normal and mature blood vessels from the surrounding healthy tissues. Achieving necrosis in the central and peripheral area is of the utmost importance [32]. It is assumed that drug-resistant cells are often localized in the central region [33] and that the viable tissue in the edge of the tumor is the preferable site of homing for bone marrow-derived circulating endothelial progenitor cells (and possibly other types of bone marrow-derived cells) when angiogenesis is triggered [34], for the reason of their proximity with blood vessels from normal tissue. Therefore, reducing mean vascular density and the viable rim area should be considered as an entwined effect. This was potentiayed in this study when tumors were treated with the vascular and tumor targeted pH-sensitive formulation containing DXR.

Furthermore, cancer cells of non-treated mice and of animals receiving free or DXR encapsulated in non-targeted (pH-sensitive or non-pH-sensitive) and targeted non-pH-sensitive liposomes were able to invade the surrounding normal mammary parenchyma and muscle fibers, whereas mice treated with the targeted pH-sensitive formulation showed no invasion whatsoever to the adjacent tissue. In this case, the tumor cell mass was confined by a fibrotic pseudocapsule. Such observation is clinically relevant, since surgical procedures and treatments are selected based on the definition of tumor “margins of resection.” These margins indicate whether all cancer cells have been removed after surgery. Although this information is not the only one influencing prognosis (likelihood of recovery without metastasis or recurrence), it is extremely important for treatment, as it increases the probability of tumors falling in the category of “negative margins,” which is associated with local control of the tumor [35], reduced risk of relapse [36] and higher overall survival [37]. These results show that F3-targeted pH-sensitive liposomes caused a major impact on tumor progression, at a microscopic level. This awareness is rather important, since a morphologic and mechanistic insight is of the utmost importance to the clinical understanding of malignancy and invasive behavior of a tumor.

**Methods**

MDA-MB-231, Hs578T, MDA-MB-435S, T47D, and MCF-7 cells were from American Type Culture Collection. HMEC-1 cells were a generous gift by the Centers for Disease Control and Prevention (Atlanta, GA). Doxorubicin hydrochloride (DXR) was from Farma-APS.
Lipids 1,2-phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (Rh-PE), used as a membrane marker at 1 mol% in cellular association experiments, dioleoylphosphatidylethanolamine (DOPE), cholesteryl hemisuccinate (CHEMS), fully hydrogenated soy phosphatidylcholine (HSPC), cholesterol (CHOL), distearoylphosphatidylethanolamine methoxy(polyethylene glycol) (2000) (DSPE-PEGM), and distearoylphosphatidylethanolamine maleimide (polyethylene glycol) (DSPE-PEGMAL) (DSPE-PEG-MAL, 4:2:2:0.18:0.12 molar ratio) were purchased from Avanti Polar Lipids. [14C]DXR and [3H]cholesteryl n-hexadecylether (CHE) were purchased from GE Healthcare. PEGm, and distearoylphosphatidylethanolamine maleimide groups were quenched by incubation with a 5-fold excess of 2-mercaptoethanol (Sigma), for 30 min at room temperature. Uncoupled peptide was separated in a Sepharose CL-4B (Sigma) column equilibrated with HBS [30]. Final lipid concentrations were determined based on the lipid phosphorous assay by Fiske and Subarrow [40]. The encapsulated DXR was quantified by measuring UV absorbance at 492 nm. Loading efficiency (%) was determined using the formula \[
\frac{[\text{DXR}/\text{Total Lipid}]}{[\text{DXR}/\text{Total Lipid}]_{\text{initial}}} \times 100.
\]

Preparation of liposomes

Non-pH-sensitive liposomes (HSPC:CHOL:DSPE-PEG:DSPE-PEG-MAL, 2:1:0.06:0.04 molar ratio) and pH-sensitive liposomes (DOPE:CHEMS:HSPC:CHOL:DSPE-PEG:DSPE-PEG-MAL, 4:2:2:0.18:0.12 molar ratio) were prepared as previously described [30, 31, 38, 39]. Both types of liposomes were extruded sequentially through polycarbonate membranes of 200 and 100 nm pore size at 65°C using a Lipofast mini extruder (Lipofast, Avestin) [38]. The lipid film was hydrated at 65°C in 250 mM ammonium sulfate solution, pH 5.5 or 8.5 for the non- and pH-sensitive formulations, respectively. The buffer was exchanged in a Sephadex G-50 (Sigma) column equilibrated with a buffer solution of 100 mM NaCH3COOH and 70 mM NaCl, pH 5.5, for the non-pH-sensitive formulation, and with 25 mM Trizmabase in 10% sucrose, pH 9, for the pH-sensitive formulation. DXR was then incubated with liposomes for 1 h at 65°C, in the absence of light and encapsulated by the ammonium sulfate gradient method [39]. Free DXR was removed by running the liposomes through a Sephadex G-50 column equilibrated with 25 mM HEPES and 140 mM NaCl buffer, pH 7.4 (HEPES buffer saline, HBS), for non-targeted liposomes, or with a buffer solution composed of 25 mM HEPES, 25 mM 2-(N-morpholino)ethanesulfonic acid (MES) and 140 mM NaCl, pH 6.5, for targeted non-pH-sensitive liposomes or of 25 mM HEPES, 25 mM MES and 140 mM NaCl, pH 7.2, for targeted pH-sensitive liposomes. To further prepare targeted liposomes, thiolated derivatives of F3 and non-specific peptide (ARALPSQRSR [16]) were obtained by the reaction of each peptide with 2-mercaptopropionimidate hydrochloride (a.k.a. 2-iminothiolane) (Sigma), in 25 mM HEPES, 140 mM NaCl buffer, pH 8, for 1 h at room temperature. Liposomes were then incubated overnight at room temperature with the thiolated peptide. Activation and covalent coupling of the peptide took place in an inert N2 atmosphere in silicon-coated glassware (Sigmacote, Sigma). Free

Immunochemistry

Tumors resected after mastectomy were fixed in Tissue Tek (Sakura), processed by standard methods, embedded in paraffin and sectioned at 3 μm. Immunohistochemistry was performed with the ABC method and staining reaction was revealed by 3,3′-diaminobenzidine tetrahydrochloride (DAB) chromogen, using DAKO automated immunostainer (Autostainer Link48 - AS480). Heat-induced antigen retrieval was conducted in PT Link PT101 (DAKO) before immunostaining. Proper positive and negative controls were selected. Sections were stained for nucleolin with biotinylated F3 peptide, estrogen (6F11, Novocastra), progesterone (1A6, Novocastra), HER2 (SP3, Dako) and EGF (AHP1372, Serotec) receptors, and for proliferation status with ki-67 (MIB-1, Dako).

Cellular association

Human breast cancer cells were harvested from tumors from patients submitted to mastectomy or tumorectomy, and cultured in FBS-enriched culture medium. Cells were incubated with F3-targeted and non-targeted rhodamine-labeled liposomes at 4 and 37°C, for 1 h, washed with PBS and immediately run in a FACSscan (Becton–Dickinson) for detecting cell-associated rhodamine (FL2-H). A total of 10,000 events were collected. Files were analyzed with Cell Quest Pro software.

In vitro studies

Cell culture

Cells were cultured in RPMI-1640 (Sigma) supplemented with 10% (v/v) heat-inactivated Foetal Bovine Serum.
(Invitrogen), 100 U/ml penicillin, and 100 µg/ml streptomycin (full medium) at 37°C in 90% humidity and 5% CO2. HMEC-1 cells medium was supplemented with 10 ng/ml mouse epidermal growth factor and 1 µg/ml hydrocortisone.

Cellular association studies

Cellular association studies were performed by flow cytometry and confocal microscopy.

Flow cytometry

To determine cellular association and payload delivery by flow cytometry, cells were incubated with rhodamine-labeled liposomes F3-targeted (SLF3), targeted by a non-specific peptide (SLNS) or non-targeted (SL), at 37 or 4°C, for 1 h. Cells were detached with dissociation buffer, washed with PBS and cell-associated rhodamine was determined by flow cytometry as previously described. A total of 40,000 events were collected.

Confocal microscopy

MDA-MB-435S or HMEC-1 cells were incubated for 1 h, at 4 or 37°C, with liposomes double-labeled with rhodamine (red label of the lipid membrane) and calcine at self-quenched concentration (green label of the aqueous core). Afterward, cells were washed, fixed with paraformaldehyde and visualized with a LSM-510 laser-scanning confocal microscope (Carl Zeiss LSM510 Meta, Zeiss), using a 488 and 561 nm excitation laser and a 63×/1.40 oil objective. Images (512 × 512 pixels) were acquired using LSM-510 software. All instrumental parameters pertaining to fluorescence detection and image analysis were held constant to allow sample comparison.

Angiogenesis assay

Matrigel-induced capillary tube formation

Two distinct assays were performed to evaluate the ability of the targeted formulation to interfere with vessel formation and disruption of already formed capillary-like structures. Aiming at evaluating the impact on the formation of new blood vessels, HMEC-1 cells were plated on GFR-Matrigel (BD Biosciences) and immediately incubated with DXR encapsulated in F3-targeted, non-specific-targeted or non-targeted pH-sensitive or non-pH-sensitive liposomes, for 24 h at 37°C.

To assess the impact on the disruption of preformed capillary-like structures, HMEC-1 cells were allowed to adhere to GFR-Matrigel before being incubated with each formulation for 16 h at 37°C. Untreated cells were used as controls. Cells were washed after incubation time was completed (24 h). Treatments were performed in triplicate. The plates were photographed before and after incubation, on an inverted phase-contrast microscope (Cannon). Percentage of vessel destabilization was determined.

Semi-quantitative measurement of tube formation in Matrigel

A semi-quantitative measurement of cord formation was performed (tube formation index) [41]. Briefly, photographs of two representative fields of each culture were obtained in a low magnification, comprising the total surface area of the well. Each cord portion between two ramifications was considered one cord unit. The number of cord-like structures was measured in each field by two observers separately, in a blinded manner. Mean values were obtained by evaluating all fields of each well under the same treatment.

Cytotoxicity studies

In vitro cytotoxicity of DXR, either free or encapsulated in liposomes, was determined for MDA-MB-435S and HMEC-1 cells using the MTT (3-(4,5-Dimethylthiazol)-2,5-diphenyltetrazolium bromide) proliferation assay, as previously described [42]. IC₅₀ of DXR mediated by the different formulations was determined from the dose–response curves at 1, 3, 24, and 48 h incubation time points.

In vivo studies

Biodistribution studies

Animal experiments were according to accepted standards of animal care (86/609/EEC and Portuguese Act 129/92). Balb/c nude (nu/nu) female mice bearing orthotopically implanted MDA-MB-435S tumors (mean volume 100 mm³) were injected via tail vein with a single dose of F3-targeted or non-targeted non-pH-sensitive or pH-sensitive liposomes (0.5 µmol phospholipid/mouse), containing approximately 1.5 × 10⁶ cpm of the lipid tracer [³H]CHE and 2.0 × 10⁵ cpm of the encapsulated drug [¹⁴C]DXR. Mice (three mice/group) were anesthetized and killed by cervical dislocation 4 and 24 h after injection. Blood was withdrawn by heart puncture, major organs were collected and radioactive labels were counted in a Beckman LS5000 TD counter. Blood correction factors were applied and biodistribution parameters determined as previously described [43]. Data are expressed as the percentage of injected dose/g of tissue.
Therapeutic studies

MDA-MB-435S-derived orthotopic tumors (eight mice/group) were allowed to grow for 25 days and systemic DXR treatment (free or encapsulated in F3-targeted or non-targeted pH-sensitive or non-pH-sensitive liposomes) was initiated (5 mg DXR/kg every week for 5 weeks). A non-treated group injected with physiological buffer was included. Tumor volume was determined twice a week. Mice were monitored routinely for physical status. Body weight losses higher than 20% were not allowed. Mice were sacrificed 24 h after the last treatment.

Tumor analysis

Organs were processed by standard methods. Under the supervision of a pathologist, histological evaluation of microscopic metastases was performed for all collected tissues (heart, lungs, liver, spleen, kidneys, brain, and bone) and characterization of the tumor with staining for nucleolin, estrogen, progesterone, HER2, and EGF receptors was conducted. Proliferation status was assessed with ki-67. H&E stained slides were visualized in a Zeiss microscope and images treated with AxioVision software for necrotic and viable area measurements. Immunohistochemical staining of tumor vessels was performed with factor VIII-related antigen (F8/86, Dako). Microvessels were counted in the most vascularized areas (200× field) and analysis was performed in a blinded manner. Data are shown as mean values ± SD of each group.

Statistical analysis

Data are given as mean ± SD in quantitative experiments. Statistical analysis of differences between experimental groups and controls were performed by one-way or two-way ANOVA. Differential findings were significant if two-tailed P values were <0.05.

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