Title: Cyclophosphamide-mediated tumor priming for enhanced delivery and anti-tumor activity of HER2-targeted liposomal doxorubicin (MM-302)

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Abstract

Given the bulky nature of nanotherapeutics relative to small molecules, it is hypothesized that effective tumor delivery and penetration are critical barriers to their clinical activity. HER2-targeted PEGylated liposomal doxorubicin (MM-302, HER2-tPLD) is an antibody-liposomal drug conjugate designed to deliver doxorubicin to HER2-overexpressing cancer cells while limiting uptake into non-target cells. In this work we demonstrate that the administration and appropriate dose sequencing of cyclophosphamide can improve subsequent MM-302 delivery and enhance anti-tumor activity in preclinical models without negatively affecting non-target tissues, such as the heart and skin. We demonstrate that this effect is critically dependent on the timing of cyclophosphamide administration. Further, the effect was found to be unique to cyclophosphamide and related analogues, and not shared by other agents, such as taxanes or eribulin, under the conditions examined. Analysis of the cyclophosphamide-treated tumors suggests the mechanism for improved MM-302 delivery involves the induction of tumor cell apoptosis, reduction of overall tumor cell density, substantial lowering of interstitial fluid pressure and increase in vascular perfusion. The novel dosing strategy for cyclophosphamide described herein is readily translatable to standard clinical regimens, represents a potentially significant advance in addressing the drug delivery challenge, and may have broad applicability for nanomedicines. This work formed the basis for clinical evaluation of cyclophosphamide for improving liposome deposition as part of an on-going Phase I clinical trial of MM-302 in HER2-positive metastatic breast cancer.
Introduction

Effective delivery of drugs to tumors is limited by distinctive features of the tumor pathophysiology. Tumor growth is supported by a rapidly growing, hyper-permeable vasculature, with blood flow abnormalities due to compression by the surrounding tumor cells (1,2). The hyper-permeability is advantageous for nanoparticle delivery (including liposomes) because it allows passive accumulation of nanoparticles in the tumor through the enhanced permeability and retention (EPR) effect (3). However, the extravasation of fluids/proteins from the leaky vasculature leads to elevation of the colloid osmotic pressure within the interstitium, a situation that is further exacerbated by the absence of functional lymphatics (4,5). As a result, the interstitial fluid pressure (IFP) increases within tumors (6–8), counteracts drug extravasation, and limits the convection process, on which efficient tumor penetration of high molecular weight drugs depends (2,9–11).

Normalization of the tumor vasculature with anti-angiogenic agents has been shown to reduce IFP and improve the activity of concomitant chemotherapy, both in preclinical tumor xenograft models as well as in metastatic rectal cancer patients (12–14). However, vascular normalization is not suitable to improve delivery of nanoparticles, likely because it decreases vascular leakage, thus compromising the EPR effect. The dual VEGF and PDGF receptors inhibitor pazopanib, while decreasing IFP and microvessel density within tumors, does not significantly affect overall accumulation of PEGylated liposomal doxorubicin (PLD) within tumors, and compromises the penetration of PLD away from tumor blood vessels (15). Similarly, normalization of tumor blood vessels with the anti-VEGFR2 antibody, DC101, increases the tumor delivery of small nanoparticles (~12 nm diameter) but reduces the delivery of large nanoparticles (~125 nm diameter) and has no effect on the anti-tumor activity of the ~100 nm diameter PLD (16). Targeting of the stroma and extracellular matrix was proven to be a more effective strategy for improving delivery and distribution of nanomedicines to tumors. Reduction of collagen content by
blockade of TGF-beta signaling, matrix degradation with hyaluronidase or inhibition of hyaluronan synthesis improve the delivery and distribution of liposomal doxorubicin (17–22). Another strategy applied to improve delivery of nanomedicines to tumors has been to directly target the tumor cell mass. Uncontrolled tumor cell growth indirectly affects vasculature function by compressing and collapsing tumor blood vessels (23,24). Treatment with taxanes, for example, results in a reduction of tumor cell mass and lowering of tumor IFP, and, as a consequence, increases PLD accumulation at the tumor site (25,26).

Preclinical findings suggest a possible role for cyclophosphamide in altering liposome delivery, via a mechanism involving endothelial cell apoptosis (27). Cyclophosphamide is a widely used drug in breast cancer therapy and combines effectively with doxorubicin. Furthermore, recent clinical data indicate that cyclophosphamide likewise combines well with liposomal doxorubicin and trastuzumab in HER2-positive breast cancer, with observed overall response rates of about 69% and good tolerability (28,29). Current clinical approaches suggest administration of cyclophosphamide and liposomal doxorubicin on the same day q3w for the treatment of breast cancer patients (28–30).

In this work we utilize MM-302 (HER2-targeted PEGylated liposomal doxorubicin, HER2-tPLD) as a model nanoparticle to study the effects of alternative cyclophosphamide regimens on nanoparticles delivery and anti-tumor activity. MM-302 is an antibody-liposomal drug conjugate engineered to selectively deliver doxorubicin to HER2 overexpressing tumor cells while sparing non-target tissues (31–33), and is being studied in a randomized Phase 2 trial in HER2-positive metastatic breast cancer (MBC) in combination with trastuzumab (http://clinicaltrials.gov/ct2/show/NCT02213744). The proposed mechanism of action of MM-302 includes 2 sequential steps: 1) extravasation from the vascular lumen into the tumor interstitium through leaky endothelium, followed by 2) internalization in HER2 expressing cells. The extravasation step is independent of the anti-HER2 targeting moiety of MM-302. Similarly to
non-targeted liposomes, MM-302 accumulates at the tumor site by passive extravasation through the EPR effect. Once MM-302 has reached the tumor interstitium, the second step occurs: HER2-mediated internalization regulates uptake into HER2 overexpressing tumor cells (33). Our studies demonstrate that cyclophosphamide, when administered in a novel pre-dosing regimen, has a unique ability to improve the delivery and anti-tumor activity of MM-302. We further demonstrate a mechanism that consists of tumor debulking, lowering of IFP and increase in vascular perfusion. In addition, cyclophosphamide effects are in contrast with observations with other chemotherapeutic agents that counterintuitively have opposite effects on MM-302 delivery under the conditions investigated.

This newly explored combination regimen of MM-302 with cyclophosphamide is currently being investigated in the clinical setting in a Phase I trial (http://clinicaltrials.gov/show/NCT01304797).
**Materials and Methods**

**Cell Culture**

NCI-N87 and MDA-MB-361 were purchased from ATCC (Manassas, VA). SUM190 were purchased from Asterand (Detroit, MI). The cell lines were cultured under recommended conditions. BT474-M3 cells are an HER2 overexpressing cell line gift from Hermes Biosciences (San Francisco, CA) and were grown in RPMI medium containing 10% FBS and 1% penicillin G/streptomycin sulphate. The cell lines were obtained between 2008 and 2011. All cell lines, with the exception of BT474-M3, were authenticated by the respective vendor (ATCC or Asterand) before receipt, and they were propagated for less than 6 months after resuscitation.

**Animal studies**

Seven-week-old female NCR/nu and nu/nu nude mice were purchased from Taconic (Germantown, NY) and Charles River Laboratories (Wilmington, MA), respectively. The care and treatment of experimental animals was in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines.

- **Establishment of xenografts and dosing:** To establish tumors, NCR/nu mice were inoculated with \(1.5 \times 10^6\) BT474-M3 cells, \(8 \times 10^6\) MDA-MB-361 cells, or \(10 \times 10^6\) SUM190 cells into the mammary fat pad (m.f.p.). Growth of estrogen-dependent models was supported by estrogen pellet implantation. Nu/nu mice were inoculated with \(5 \times 10^6\) NCI-N87 cells (s.c. into the left and right flank of the mouse). When tumor volume reached 200-300 mm\(^3\), mice were dosed with PBS or with cyclophosphamide (170 mg/kg, intraperitoneally (IP) at the designated time points. Alternatively, ifosfamide (150 mg/kg, IP), paclitaxel (40 mg/kg, intravenously (IV)) or eribulin (0.45 mg/kg, IV) were given 7 days prior to HER2-tPLD (3 mg/kg doxorubicin equivalents, IV). Cyclophosphamide, eribulin and ifosfamide were each dissolved in PBS. Paclitaxel was dissolved in Cremophor:ethanol (1:1) and further diluted 1:4 with PBS (final Cremophor
content of 12.5%), immediately prior to administration. Mice were subsequently dosed IV with fluorescently labeled HER2-tPLD (DiI5-labeled) (3 mg/kg) or free doxorubicin (3 mg/kg), as indicated. Following liposome (24 h) or free doxorubicin injection (30 min or 24 h), and 5 min before sacrificing, mice received 200 ul of FITC-lectin (IV) to label the perfused vasculature. Immediately after sacrificing, mice were perfused with PBS to remove liposomes still in circulation.

- **Tumor growth inhibition**: When tumor volume (width$^2 \times$length$\times$0.52) reached $\sim$320 mm$^3$ the mice were randomized into groups of 10 mice/group that received: PBS (control), HER2-tPLD (3 mg/kg, IV q7d; three total doses) or cyclophosphamide (170 mg/kg, IP q14d). Two additional groups received a combination of cyclophosphamide (170 mg/kg, IP) dosed 96 h prior to the first and the third dose of HER2-tPLD or a combination of cyclophosphamide (170 mg/kg, IP) dosed simultaneously with the first and the third dose of HER2-tPLD (3 mg/kg IV). Tumor growth and changes in mouse weight were monitored twice/week. Tumor growth inhibition (TGI) was calculated by subtracting the average % tumor growth of the group investigated from the average % tumor growth of the control group and then by dividing the result by the average % tumor growth of the control group.

- **Interstitial fluid pressure (IFP) measurements within tumors**: IFP measurements were performed as described in Ozerdem U., et al (34). Mice were sedated with isoflurane and the tumor surface was disinfected. After pressure calibration, the tip of a catheter (SPR-320, ADInstruments, Inc., Colorado Springs, CO) was inserted into a sterile 18G needle that, in turn, was inserted into the mouse tumor for IFP measurement. After insertion in the tumor mass, the needle was retracted, the pressure was allowed to equilibrate and finally the intratumoral pressure was recorded for an average of 2 min with the LabChart software (ADInstruments, Inc., Colorado Springs, CO). IFP was measured in an average of 5 different locations/tumor.

**Radiolabeling of HER2-tPLD for PET Imaging**

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HER2-tPLD was labeled with $^{64}$Cu for positron emission tomography (PET) imaging. Detailed development and characterization of $^{64}$Cu-HER2-tPLD as a liposomal PET agent was described elsewhere (35). Briefly, $^{64}$CuCl$_2$ was added to the chelator, 4-DEAP-ATSC, in 0.1 M citrate buffer, pH 6 (~ 0.35 mCi/nmol chelator) and mixed at RT for 1 min. Formation of the $^{64}$Cu:4-DEAP-ATSC complex was confirmed using instant thin layer chromatography (ITLC) with chelation efficiency >98%. The $^{64}$Cu:4-DEAP-ATSC complex was then loaded into HER2-tPLD by incubating at 65°C for 10 min. Loading of $^{64}$Cu:4-DEAP-ATSC into HER2-tPLD was confirmed using size exclusion chromatography (SEC), at a loading efficiency of >90%.

**Non-Invasive PET/CT imaging of HER2-tPLD tumor deposition**

BT474-M3 tumor-bearing mice were randomized into a PBS-treated group (n= 6) and a cyclophosphamide-treated group (n= 6). All mice were injected IV with the liposomes described below at a total lipid dose of 20 umol/kg (3 mg/kg HER2-tPLD equivalent). At day 0, mice were injected with a trace dose of $^{64}$Cu-HER2-tPLD mixed with drug-free-HER2-tPLD (20%/80% v/v, respectively) in order to maintain the total lipid dose and to minimize potential treatment effects (“$^{64}$Cu-HER2-tPLD-trace”). At 20 h post-injection (day 1), mice were imaged (baseline tumor deposition). On day 3, mice were injected IP with PBS or 170 mg/kg of cyclophosphamide. On day 8, mice were injected with a full dose of $^{64}$Cu-MM-302 and were imaged 20 h post-injection (day 9, post-treatment tumor deposition). Two weeks later (week 5) the experiment was repeated.

**PET/CT Image Acquisition and Tumor Deposition Quantification**

PET images were acquired using a Focus 220 microPET (Siemens; Malvern, PA). PET data were reconstructed using Siemens’s 3D ordered subset expectation maximization and a maximum a posteriori (OSEM3D/MAP) algorithm (with scatter and attenuation correction) with a voxel size of 0.32 x 0.32 x 0.8
mm³. Computer tomography (CT) scans were acquired using a Locus Ultra microCT (GE Healthcare; Pittsburgh, PA) operating at 80 kVp and 50 mA and an isotropic voxel size of 154 µm. PET/CT images were registered and analyzed using the Inveon Research Workplace software (IRW, Siemens; Malvern, PA). The mean tumor deposition of ⁶⁴Cu-MM-302 was measured from the contoured regions-of-interest (ROIs).

**Quantification of doxorubicin within tissues**

Quantification of doxorubicin within tumors, hearts and dorsal skin was performed by HPLC as previously described (32).

**Immunofluorescence**

Immediately after collection, one part of each tumor was fixed in 10% neutral buffered formalin to generate formalin-fixed paraffin-embedded slides (5-µm thick). An additional tumor piece was embedded in OCT (optimal cutting temperature) compound, and further processed to cryosections (10-µm thick). Cleaved caspase 3, γ-H2AX, cleaved PARP, HER2 and cytokeratin stainings were performed as described in (36). For collagen type I and CD31 stainings slides were air-dried and fixed in 10% neutral-buffered formalin (20 min at RT). A summary of the reagents and methods is included in Supplementary Table S3. DiI5-labelled liposomes, doxorubicin and perfused vessels (FITC-lectin labelled) were imaged on unfixed frozen tumor sections on an Aperio Scanscope FL (Aperio, Vista, CA) as described in (32).

**Image analysis of immunofluorescence**

Images were analyzed using custom rulesets in Definiens Developer XD 2 (Definiens, Munich, Germany) as described in the Supplementary Material. The whole cross section of the individual tumors were analyzed (1 level/tumor).
Statistical analysis

The data were analyzed with the software GraphPad Prism version 6.0 (La Jolla, CA). Results were analyzed by non-parametric unpaired two-tailed Student’s t test (Mann-Whitney). Alternatively, for the analysis of the IFP measurements, results were generated in SAS® version 9.3 (Cary, NC) using a randomized block analysis of variance model for pressure with treatment as a fixed effect and blocks (mice) as a random effect in the model. The data is shown as mean ± standard deviation (SD) when not differently indicated.
Results

Effects of Cyclophosphamide on delivery of HER2-tPLD and free doxorubicin

BT474-M3 tumor-bearing mice were predosed with cyclophosphamide (170 mg/kg) 2-5 days prior to injection of HER2-tPLD or free doxorubicin (both at 3 mg/kg). The doxorubicin content within individual tumors, dorsal skin and hearts was quantified by HPLC and expressed as percentage of injected dose per gram of tissue (% i.d./g) (Fig. 1A-C). Predose with cyclophosphamide had no effect on the delivery of free doxorubicin, but significantly increased (2-3-fold) the tumor delivery of HER2-tPLD in a dose dependent fashion (Fig. 1A and Supplementary Fig. S1A). The predose timing was critical. Indeed, co-injection of cyclophosphamide with HER2-tPLD showed only a mild trend toward increasing delivery of HER2-tPLD to tumors (Supplementary Fig. S1B). The tumor delivery of PLD was also increased by predose with cyclophosphamide in both BT474-M3 and SUM190 tumors (Supplementary Fig. S1C-D). Pharmacokinetic studies confirmed that the increase in HER2 t-PLD tumor delivery was not a result of a delay of HER2 t-PLD blood clearance (Supplementary Fig. S1E). Importantly, the increase in delivery of HER2-tPLD was tumor-specific as no change in doxorubicin deposition was detected in dorsal skin or heart tissue (Fig. 1B and 1C).

The ratios of %i.d./g tissue in matched tumors and heart tissues were calculated for HER2-tPLD (24 h) and free doxorubicin (30 min). The 30 min time point was chosen for free doxorubicin because, while the levels of free doxorubicin in tumors are similar at 30 min and 24h, the 30min time point is a better representative of the peak delivery of free doxorubicin to the heart tissue (32). Pre-dosing with cyclophosphamide resulted in a significant improvement in the tumor/heart delivery ratio for HER2-tPLD (Fig. 1D). Conversely, the tumor/heart delivery ratio remained unchanged for free doxorubicin (Fig. 1D).

Effects of multiple cycles of cyclophosphamide treatment on intra-animal tumor deposition
PET/CT imaging studies were performed to monitor cyclophosphamide-induced changes in $^{64}$Cu-HER2-tPLD deposition within an individual tumor as described in Fig. 2A. BT474-M3-tumor bearing mice were injected with $^{64}$Cu-HER2-tPLD-trace, containing trace amounts of doxorubicin to minimize effects on the tumor microenvironment. Baseline deposition of HER2-tPLD was assessed by PET/CT imaging 20 h post-liposome injection (day 1). Subsequently (day 3), mice received either PBS (control group, n=5) or a single dose of cyclophosphamide (170mg/kg, n=6) and changes in deposition relative to day 1 were recorded by PET/CT scans on day 9, 20 h after injection of $^{64}$Cu-HER2-tPLD (end of cycle 1). After a washout period of about 2 weeks, the above was repeated for a second cycle of PBS or cyclophosphamide treatment. Maximum intensity projection (MIP) and axial views of representative mice of both groups for week 1 and week 2 are shown in Fig. 2B. An increase in total $^{64}$Cu-HER2-tPLD tumor deposition can be observed in the cyclophosphamide pre-treated mouse at week 2 relative to week 1, but not in the PBS-treated mouse (Fig. 2B). Tumor deposition of $^{64}$Cu-MM-302 was quantified in each individual mouse by region-of-interest (ROI) analysis. Significant increases in tumor deposition at week 2 relative to week 1 and at week 6 relative to week 5 were observed in cyclophosphamide-treated mice but not in control mice (Supplementary Table S1). The percent changes in tumor deposition at week 2 relative to week 1 (cycle 1) and at week 6 relative to week 5 (cycle 2) were calculated for the individual mice (Fig. 2C). A significant increase in tumor deposition relative to the PBS-treated group at both cycle 1 and 2 was observed in the cyclophosphamide-treated mice, indicating that the cyclophosphamide effects on HER2-tPLD deposition persist and can be detected in subsequent cycles if a pre-dose is applied.

_Cyclophosphamide-mediated changes in the tumor microenvironment_

To understand the mechanism by which cyclophosphamide increases HER2-tPLD tumor delivery, BT474-M3 tumor sections were analyzed by histology. HER2-tPLD accumulation at the tumor site is
driven by EPR, and is minimally affected by HER2 expression (33). To rule out increased HER2-tPLD retention at the tumor site via increased HER2 expression, HER2 was quantified by staining and quantification as in (36). Cyclophosphamide did not alter HER2 expression (receptor numbers/cell) (Supplementary Table S2).

To detect DNA damage/response, BT474-M3 tumors were stained with an anti-\(\gamma\)-H2AX antibody and images were analyzed. The extent of \(\gamma\)-H2AX-positive cells increased as early as 2 days post-cyclophosphamide dose, peaked at 4 days, and started to decrease 5 days post-cyclophosphamide dose (Supplementary Fig. S2A). DNA damage was accompanied by apoptosis, detected by an antibody against cleaved caspase 3 (Supplementary Fig. S2B), with a timing similar to that observed for \(\gamma\)-H2AX. In addition, apoptosis was also detected with an anti-cleaved PARP antibody coupled with cytokeratin staining to specifically detect apoptosis in tumor cells (cytokeratin positive). The results showed a similar dynamics as observed for cleaved caspase 3 (Supplementary Fig. S2C). As a result of persistent tumor cell apoptosis, cyclophosphamide caused significant changes in tumor cell density relative to control tumors (Fig. 3A). Changes in interstitial space within tumors, defined as the area not occupied by any cellular component, were also quantified (Fig. 3B). Treatment with cyclophosphamide resulted in a significant increase (up to 1.6-fold) in interstitial space area.

The above effects could result in changes in interstitial fluid pressure (IFP) that ultimately could account for the improved delivery of HER2-tPLD. IFP measurements were carried out in BT474-M3-tumor-bearing mice to test this hypothesis (Fig. 3C). Treatment with cyclophosphamide (4 days) resulted in a ~36% decrease in IFP (10.6 ± 2 mmHg) compared to the control group (16.6 ± 4.7 mmHg).
Modifications of the extracellular matrix to reduce collagen deposition have been shown to significantly improve deposition of nanoparticles (4,17,18,37). Cyclophosphamide however did not alter the deposition of collagen type I in BT474-M3 tumors (Supplementary Table S2).

We subsequently investigated the effects of cyclophosphamide on the vasculature of BT474-M3 tumors. Changes in microvessel density (MVD) were evaluated by CD31 immunostaining. Cyclophosphamide did not induce any changes in MVD (Supplementary Table 2). Besides MVD, the extent of vascular perfusion was quantified, and expressed as % of FITC-lectin positive vessels relative to the total CD31 positive vessels. Control BT474-M3 tumors showed a relatively high extent of perfusion (85.03%) (Supplementary Table S2). The small blood vessels were characterized by the lowest extent of perfusion in the control tumors (64.92%) followed by the medium (85.81%) and the large blood vessels (96.52%). After cyclophosphamide treatment (4 days), an increase in perfusion was observed in all three populations with more prominent changes in the small blood vessels (15.5%), followed by the medium (3.72%) and the large blood vessels (1.61%) (Fig. 3D; Supplementary Table 2).

*Only cyclophosphamide and its analogue, ifosfamide, increase HER2-tPLD delivery*

We next investigated whether other chemotherapeutic agents would elicit changes in HER2-tPLD delivery. The cyclophosphamide analogue, ifosfamide, and two microtubule inhibitors, paclitaxel and eribulin, were tested. BT474-M3-tumor bearing mice (bilateral tumors) were treated with PBS or dosed with the above agents, and HER2-tPLD was subsequently administered. Tumors were collected 24 h post-HER2-tPLD injection and doxorubicin was quantified by HPLC (Fig. 4A). Ifosfamide (I) increased delivery of HER2-tPLD similarly to cyclophosphamide (~2-fold). No changes in delivery were observed following pretreatment with eribulin (E). Surprisingly, pretreatment with paclitaxel (P) reduced delivery of HER2-tPLD. Similar results were observed with paclitaxel in an additional tumor model (Supplementary Fig. S3A). The effects of paclitaxel were driven by its vehicle Cremophor, as Cremophor
alone caused a similar response. The negative effects of paclitaxel on HER2-tPLD delivery persisted for up to three weeks post paclitaxel administration (Supplementary Fig. S3B).

To gain insight into comparative mechanisms for the other agents tested, matched FFPE tumor sections were stained for cytokeratin to quantify tumor cell density (Fig. 4B-C). Consistent with the observations with cyclophosphamide, its analogue ifosfamide (I) also elicited a significant reduction in tumor cell density, whereas no changes were observed with paclitaxel (P). Even though eribulin (E) did not have any effects on delivery, the tumor cell density of eribulin-treated tumors was markedly reduced. However, eribulin dramatically increased stromal cells density (~8-fold) (Fig. 4D). Eribulin induced similar changes in an additional xenograft model (Supplementary Fig. S3C). Cyclophosphamide, ifosfamide and paclitaxel had no significant effects on stromal cell density. As a consequence, the overall total cell density was significantly reduced only in the cyclophosphamide and ifosfamide-treated tumors, but not in the eribulin or paclitaxel-treated tumors (Supplementary Fig. S3D). Similarly, increases in interstitial space area were observed with both cyclophosphamide and ifosfamide, but not with eribulin and paclitaxel (Supplementary Fig. S3E).

Effects of cyclophosphamide on liposome distribution and nuclear doxorubicin

To gain information on the effects of cyclophosphamide on the intratumoral distribution of HER2-tPLD, frozen tumor sections from DiI5-HER2-tPLD-injected BT474-M3 tumors were imaged (Fig. 5A). Predose with cyclophosphamide resulted in a significant increase in DiI5-liposome signal, which was proportional to the total doxorubicin determined by HPLC in matched tumors (linear regression fit, R²=0.8164) (Supplementary Fig. S4A). Changes in the spatial distribution of liposomes relative to the vasculature were quantified. Perfused tumor blood vessels were identified based on the lectin signal and were classified into “Low Delivering” (LD) or “High Delivering” (HD) vessels based on the DiI5-liposome MFI within the vessels as described in the Supplementary Materials and Methods. The relative
percentages of LD and HD vessels in the control and the cyclophosphamide-treated groups were strikingly different. The HER2-tPLD alone group consisted of a majority of LD vessels (74.5 ± 15.4 %) and only 25.5 ± 15.4 % of HD vessels (Fig. 5B). Conversely, in the cyclophosphamide-treated group these relative percentages were reversed, showing 81.7 ± 11.4 % of HD vessels and 18.3 ± 11.4 % of LD vessels (Fig. 5B). We next quantified the Dil5-liposome MFI in vessel-concentric objects starting from the signal within the actual blood vessels up to 100 um away from the blood vessels (Fig. 5C). Cyclophosphamide induced a significant increase of Dil5-liposome MFI at each of the distances from blood vessels analyzed.

The percent of doxorubicin-positive nuclei at 24 h post-liposome injection was quantified (Fig. 5D). The percent of doxorubicin-positive nuclei increased 12-fold, from 4.4 ± 4.4 % to 53.7 ± 18.8 % for the cyclophosphamide-treated group. The peak in doxorubicin positive nuclei accumulation (~30um away from the nearest blood vessels) was unaffected by cyclophosphamide (Supplementary Fig. S4B). However the magnitude of doxorubicin positive nuclei away from the vasculature was significantly increased by cyclophosphamide (Supplementary Fig. S4B).

Cyclophosphamide-induced changes in HER2-tPLD doxorubicin delivery were associated with decreases in tumor cell density and enhanced nuclear delivery of doxorubicin in additional xenograft models, SUM190 and NCI-N87 (Supplementary Fig. S5). Interestingly, when no significant changes in delivery were observed, the tumor cell density and nuclear doxorubicin were also unaffected (Supplementary Fig. S5, MDA-MB-361). The MDA-MB-361 cell line was the least sensitive to cyclophosphamide in vitro (Supplementary Fig. S5D).

Pre-dosing of cyclophosphamide in combination with HER2-tPLD results in increased apoptosis and anti-tumor activity
We next investigated whether the cyclophosphamide-induced increase in nuclear delivery of doxorubicin would result in enhanced activation of downstream markers of doxorubicin-induced cytotoxicity. BT474-M3 tumor sections that were either predosed with PBS or cyclophosphamide (for 4 days) prior to HER2-tPLD treatment (24 h) were stained and analyzed to determine the percent of γ-H2AX and cleaved caspase 3 positive cells (Fig. 6A, representative fields). The HER2-tPLD and cyclophosphamide combination group induced a greater DNA damage and apoptotic response compared to both the single agent treatment groups (Fig. 6B-C).

Finally, we investigated whether the increases in cytotoxicity in the HER2-tPLD and cyclophosphamide combination group would translate into increased anti-tumor activity. BT474-M3 tumor-bearing mice were dosed with either PBS, HER2-tPLD alone (3 mg/kg), cyclophosphamide alone (170 mg/kg), or HER2-tPLD (3 mg/kg) and cyclophosphamide (170 mg/kg, given 4 days prior to HER2-tPLD) (Fig. 6D). The combination group was more effective in inhibiting tumor growth than either cyclophosphamide or HER2-tPLD alone (Fig. 6D). The combination group that received cyclophosphamide before HER2-tPLD showed a significantly higher tumor growth inhibition compared to a combination group that simultaneously received HER2-tPLD and cyclophosphamide (Fig. 6E). The effects of the novel sequential combination of cyclophosphamide with HER2-tPLD on tumor growth inhibition (TGI) were compared to that expected by Bliss Independence (i.e. assuming the drugs have non-overlapping mechanisms of action) (38). TGI at day-55 was 46.6% greater-than-additive, indicating synergy between cyclophosphamide and HER2-tPLD (Supplementary Fig. S6A).
Discussion

In this work we address one of the most significant barriers to effective treatment with nanotherapeutics, namely tumor delivery and penetration. This work is presented in the context of HER2-tPLD, an antibody liposomal drug conjugate, currently being evaluated in a randomized Phase 2 trial in combination with trastuzumab in HER2-positive MBC (39,40). In this clinical context, cyclophosphamide is an important candidate among possible combination partners because of its well-established effectiveness in combination with the free drug doxorubicin for the treatment of early-stage as well as metastatic breast cancer, both in HER2 positive and HER2 negative disease (41,42). More recently, cyclophosphamide has also shown promising clinical responses when combined with PLD in HER2 negative metastatic breast cancer patients (30) as well as in HER2 positive metastatic breast cancer patients in combination with PLD and trastuzumab (28,29).

Because of their large size, tumor delivery of nanotherapeutics, including liposomes, is restricted. This is a result of the tumor pathophysiology, characterized by elevated IFP that interferes with drug extravasation and penetration. For this reason, methods to enhance nanoparticle deposition within tumors are of great clinical value. Data presented here illustrate that, when administered in a novel ‘predose’ regimen, cyclophosphamide significantly improved the tumor delivery of HER2-tPLD in multiple preclinical models. The effect of cyclophosphamide was specific to HER2-tPLD tumor delivery, and left the delivery of free doxorubicin unaffected, suggesting that a cyclophosphamide plus HER2-tPLD combination would result in a synergistic improvement beyond that expected by the cyclophosphamide plus doxorubicin combination currently in clinical use. Importantly, the improvement in delivery was shown to be tumor-specific. Heart tissue and skin, two off-target organs of safety concern for doxorubicin as a free drug and in liposomal form, were unaffected by the predose with cyclophosphamide. The improvement in delivery of HER2-tPLD by cyclophosphamide was detected by
PET/CT imaging studies on multiple subsequent cycles of treatment and was shown to translate into improved anti-tumor activity.

The timing of cyclophosphamide administration was critical to its activity as deposition-enhancing agent. For example, co-injection of cyclophosphamide induced a non-significant increase in liposome delivery. Conversely, statistically significant increases in HER2-tPLD tumor delivery were observed in multiple models when cyclophosphamide was predosed 2 to 7 days prior to HER2-tPLD administration. These results are consistent with our proposed mechanism of reduction of tumor cell density and lowering of IFP. Our data are in slight contrast with previous reports showing that a co-injection of cyclophosphamide was able to induce a change in delivery of liposomal doxorubicin (27). Diverse sensitivities of the tumor models to cyclophosphamide, variations in tumor volumes on the day of the experiment and distinct administration modalities could account for the observed differences. Our results showed variable times required for the different models to respond to cyclophosphamide. BT474-M3 tumors responded quite rapidly to the cyclophosphamide treatment, with changes in delivery detectable as early as 2 days post-cyclophosphamide; other models required longer times for cyclophosphamide to exert an effect. In general, the window of activity for the predose peaked between 4 and 7 days, and it is expected that a similar timeframe may apply to human tumors.

The studies presented in this work indicate that the mechanism of cyclophosphamide’s deposition enhancement involves reduction of tumor cell density via apoptosis, lowering of IFP and increase in interstitial space. Further support of this mechanism is the fact that in the MDA-MB-361 model, where the deposition of HER2-tPLD remained unchanged by cyclophosphamide, the tumor cell density was likewise unaffected. Of note, among all models tested, the MDA-MB-361 cell line was the least sensitive to cyclophosphamide in vitro. The alleviation of solid stress was accompanied by a
modest increase in vascular perfusion, more pronounced on small blood vessel, which is in concordance with previous observations (16,24,43,44).

The changes in delivery of HER2-tPLD were not mediated by changes in HER2 expression at the tumor site. Furthermore, beside HER2-tPLD, cyclophosphamide also increased delivery of PLD in the HER2 positive tumor models analyzed. Taken together, these observations support a mechanism of action for cyclophosphamide-induced changes in delivery that is independent of HER2-targeting. Hence our data suggests that cyclophosphamide would have similar effects on HER2-tPLD or PLD also on HER2 negative models, as long as the models would respond to cyclophosphamide with the microenvironment changes described herein.

Based on the data thus far and proposed mechanism(s), it is likely that the effects of cyclophosphamide would extend to diverse nanoparticle types carrying different payloads (chemotherapies, siRNAs, etc.). Indeed, cyclophosphamide also increased tumor delivery of PLD. Hence timed pre-administration of cyclophosphamide may provide a general means to improve nanoparticle delivery to solid tumors.

Our analysis was expanded on other chemotherapeutic agents, well reported to have significant cell killing effects. Ifosfamide, a structural analogue of cyclophosphamide, behaved similarly to cyclophosphamide, and induced a significant increase in HER2-tPLD delivery. Treatment with microtubule inhibitors, such as paclitaxel or eribulin, resulted in no increase in nanoparticle deposition in the models tested. It should be acknowledged that a portion of our data are in contrast with previously reported results that showed that paclitaxel was able to elicit a reduction in cell density and an increase in delivery of PLD (26). In our studies, using different tumor models than the one reported by Lu et al. (26), paclitaxel significantly reduced HER2-tPLD delivery and did not reduce tumor cell density. Importantly, the baseline delivery of HER2-tPLD was not restored up to 3 weeks following
paclitaxel administration. In the two xenograft models tested, eribulin induced a non-significant reduction in delivery of HER2-tPLD. In both models eribulin dramatically increased the stromal cell density, which could explain the negative trends on delivery. The observations on paclitaxel and eribulin predoses on HER2-tPLD delivery require further investigation, and suggest that attention be paid in the clinical setting to the sequential dosing and timing of these agents relative to nanotherapeutics to avoid undesirable antagonism.

Platinum-based agents share with cyclophosphamide a DNA-damage mechanism, and they have been associated with high response rates when sequentially combined with PLD for the treatment of ovarian cancer (45). On-going research is being performed to evaluate whether an increase in HER2-tPLD or PLD delivery upon platinum-based agents treatment might be among the mechanisms underlying the high response rates in the clinic.

Delays in clearance of PLD have been observed clinically when PLD was combined with taxanes (46–48). Cyclophosphamide, particularly at high doses, is known to have also immunological effects, and therefore could alter the pharmacokinetics of subsequently administered agents. Our results indicated that cyclophosphamide had no observable effect on HER2-tPLD pharmacokinetics, thus confirming that the measured changes in delivery are associated with modifications in the tumor microenvironment, rather than with a delay in clearance of HER2-tPLD. Our data are consistent with clinical data showing that cyclophosphamide and its analogue ifosfamide have no influence on the pharmacokinetics of PLD (49,50). In addition, the animal models used in these studies are partially immuno-compromised mice, further suggesting a non-immunological based mechanism of tumor deposition enhancement.

In conclusion, our data showed that carefully timed pre-administration of cyclophosphamide has the unique property of enhancing HER2-tPLD delivery and subsequent anti-tumor activity in preclinical models. This combination strategy is readily translatable into standard clinical regimens and the clinical
utility of cyclophosphamide pre-dosing for enhancing tumor deposition of HER2-tPLD is currently being tested as part of an on-going clinical trial. Tumor deposition, assessed by $^{64}$Cu-labeled-MM-302 and PET/CT imaging, is being measured in patients with and without pretreatment with cyclophosphamide. The extent of tumor deposition will then be related to clinical measures of response at the individual lesion and patient level.
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References


Figure Legends

Fig. 1. Pretreatment of tumors with cyclophosphamide enhances the delivery of HER2-tPLD and not of free doxorubicin to BT474-M3 tumors. Mice (n=5/group) were predosed with cyclophosphamide (170 mg/kg, IP) 2-5 days prior to injection of HER2-tPLD (3 mg/kg; blue squares) or free doxorubicin (3 mg/kg; red triangles). 24 h post-liposome injection, and 30 min and 24 h post-free doxorubicin injection, mice were sacrificed and tumors (A), dorsal skin (B) or hearts (C) were collected and analyzed by HPLC to determine the total doxorubicin content expressed as %i.d./g tissue. (D) The ratios of %i.d./g tissue in matched tumors and heart tissues are shown for HER2-tPLD and free doxorubicin.

Fig. 2. Changes in delivery induced by cyclophosphamide can be detected at the individual mouse level and persist at multiple cycles. (A) Experimental outline of PET/CT imaging study to evaluate changes in HER2-tPLD delivery in individual animals. The details are described in Materials and Methods. “$^{64}$Cu-HER2-tPLD-trace” represents a trace dose of $^{64}$Cu-HER2-tPLD mixed with drug-free-HER2-tPLD at a 20%/80% v/v ratio. (B) BT474-M3 tumor bearing mice were randomized into two groups, injected with “$^{64}$Cu-HER2-tPLD-trace”, and were PET/CT imaged 20 h post-injection for measurement of the baseline tumor deposition (week 1). Afterwards, mice received either PBS (CTL, n=5) or cyclophosphamide (n=6), and 5 days later were injected with $^{64}$Cu-HER2-tPLD for a second PET/CT scan (week 2). Maximum intensity projection (MIP), and axial views images of representative mice of CTL (left panels) and cyclophosphamide-treated (right panels) groups are shown at baseline (week 1) and after treatment (week 2). White arrowheads and dashed-red circles highlight the tumors in the MIP and the axial views, respectively. (C) The percent change in tumor deposition at cycle 1 and cycle 2 for both control (CTL) and cyclophosphamide treated (C) groups is shown.
Fig. 3. Cyclophosphamide induces a reduction in tumor cell density and IFP, and increases vascular perfusion. BT474-M3 tumor bearing mice (n=5/group) were dosed with cyclophosphamide (170 mg/kg, IP) and tumors were collected 2-5 days post-injection. Tumor sections were stained for cytokeratin to label tumor cells. Slides were scanned and images were analyzed to quantify changes in tumor cell density (A). (B) Quantification of changes in interstitial space area (percent of total tumor area) upon treatment with cyclophosphamide. (C) Interstitial fluid pressure (IFP) was measured on mice treated for 4 days with either PBS (CTL, n=6) or with cyclophosphamide (170 mg/kg, IP; C; n=5). (D) Tumor sections from mice treated for 4 days with PBS (CTL) or cyclophosphamide (C) were stained for CD31, imaged for CD31 and FITC-lectin, and quantified to determine the % of perfused (green) and non-perfused (red) small (left), medium (middle) and large (right) blood vessels.

Fig. 4. The effects on delivery of HER2-tPLD, mediated by a reduction in tumor cell density, are specific for cyclophosphamide and its analogue, ifosfamide. BT474-M3 tumor bearing mice (n=4/group; bilateral tumors) were predosed either with PBS (blue squares), cyclophosphamide (C, dark blue diamonds), ifosfamide (I, magenta diamonds), paclitaxel (P, green triangles) or eribulin (E, orange circles) prior to injection of HER2-tPLD (3 mg/kg), as described in Materials and Methods. 24 h post-liposome injection, tumors were collected and processed for quantification of doxorubicin by HPLC (A). (B) FFPE-tumor sections, generated from the tumors described above, were stained for cytokeratin, counterstained with Hoechst to label the nuclei, and scanned on an Aperio Scanscope FL. Representative fields of view of the original images are shown in the top panels (cytokeratin, green; Hoechst, blue). Images were subsequently processed by segmentation to identify individual objects. The results of the segmentation are shown in the bottom panels: tumor cells (red), stromal cells (cyan) and interstitial space (black) (scale bar = 200 um). The changes in tumor cell density and stromal cell density are shown in C and D, respectively (black circles=control (CTL) tumors; mice received PBS only, no chemotherapeutic agent and no HER2-tPLD).
Fig. 5. Pretreatment of tumors with cyclophosphamide significantly enhances liposome penetration and nuclear delivery of doxorubicin following HER2-tPLD injection. BT474-M3 tumor bearing mice (n=5/group) were dosed with PBS (A, CTL, top panels) or with cyclophosphamide (170 mg/kg, IP) (A, bottom panels) 4 days before injection of fluorescently labelled HER2-tPLD (3 mg/kg) (DiI5-HER2-tPLD). Tumors were collected 24 h post-liposome injection, prepared for cryosections, counterstained with Hoechst, and scanned to visualize the microdistribution of DiI5-liposomes and doxorubicin relative to the vasculature, labeled with FITC-lectin (A, scale bar = 200 um). Images were analyzed by segmentation to identify doxorubicin positive nuclei (magenta), doxorubicin negative nuclei (blue), and blood vessels (green) (right panels). (B) The relative percentages of “Low Delivering” (LD) and “High Delivering” (HD) vessels in the HER2-tPLD (light blue) and HER2-tPLD + C group (dark blue) are shown. (C) Quantification of the DiI5-liposome MFI away from the blood vessels for the HER2-tPLD (light blue) and HER2-tPLD + C (dark blue) groups. HD and LD vessels are shown in solid and dashed lines, respectively. (D) Quantification of the percent of doxorubicin positive nuclei for HER2-tPLD (light blue), and HER2-tPLD + C (dark blue).

Fig. 6. Predose of BT474-M3 tumors with cyclophosphamide results in increased DNA damage/repair response, apoptosis and tumor growth inhibition. Mice (n=5/group) were inoculated with BT474-M3 tumor cells, followed by treatment with PBS (CTL), HER2-tPLD, cyclophosphamide (C) or a combination of the two agents (C given 4 days prior to HER2-tPLD, HER2-tPLD + C). (A) Tumors were collected 24 h post-liposome injection, prepared for FFPE, and tumor sections were stained for γ-H2AX and cleaved caspase 3 and nuclei were counterstained with Hoechst. Representative fields of view of the original images are shown in the top panels (γ-H2AX, green; cleaved caspase 3, red; Hoechst, blue). Slides were scanned and images were analyzed by segmentation to identify γ-H2AX (green), cleaved caspase 3 positive cells (pink), and double positive cells (red). The results of the segmentation are shown in the bottom panels (scale bar = 100 um). The results of the quantification of the percent of γ-H2AX and
cleaved caspase3 positive cells are shown in (B) and (C), respectively. (D) The percent of tumor growth relative to the day of randomization (day 14) is shown for the different treatment groups (n=10/group). (E) The anti-tumor activities of the single agents (cyclophosphamide and HER2-tPLD) and of two combination groups, one that received cyclophosphamide 4 days prior to HER2-tPLD and one that received cyclophosphamide simultaneously with HER2-tPLD, are shown (n=10/group). The data is presented as mean ± SEM in panel D and E.
Figure 2

A

\[ \Delta \text{Deposition cycle 1} \]

B

C

\begin{itemize}
  \item Cycle 1: p=0.0043, **
  \item Cycle 2: p=0.0238, *
\end{itemize}
Molecular Cancer Therapeutics

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