Impact of Tumor HER2/ERBB2 Expression Level on HER2-Targeted Liposomal Doxorubicin-Mediated Drug Delivery: Multiple Low-Affinity Interactions Lead to a Threshold Effect

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Abstract

Numerous targeted nanotherapeutics have been described for potential treatment of solid tumors. Although attention has focused on antigen selection and molecular design of these systems, there has been comparatively little study of how cellular heterogeneity influences interaction of targeted nanoparticles with tumor cells. Antigens, such as HER2/ERBB2, are heterogeneously expressed across different indications, across patients, and within individual tumors. Furthermore, antigen expression in nontarget tissues necessitates optimization of the therapeutic window. Understanding the performance of a given nanoparticle under different regimens of antigen expression has the ability to inform patient selection and clinical development decisions. In this work, HER2-targeted liposomal doxorubicin was used as a model-targeted nanoparticle to quantitatively investigate the effect of HER2 expression levels on delivery of doxorubicin to the nucleus. We find quantitatively greater nuclear doxorubicin delivery with increasing HER2 expression, exhibiting a threshold effect at approximately $2 \times 10^5$ HER2 receptors/cell. Kinetic modeling indicated that the threshold effect arises from multiple low-affinity interactions between the targeted liposome and HER2. These results support previous data showing little or no uptake into human cardiomyocytes, which express levels of HER2 below the threshold. Finally, these results suggest that HER2-targeted liposomal doxorubicin may effectively target tumors that fall below traditional definitions of HER2-positive tumors, thereby expanding the potential population of patients that might benefit from this agent. Mol Cancer Ther; 12(9); 1816–28. © 2013 AACR.

Introduction

Numerous targeted nanoparticles against different antigens have been described in the literature for potential treatment of solid tumors (1–12). The majority of those studies focused on the selection and design of nanoparticles and/or targeting moieties. In contrast, the relationship between antigen expression level and the performance characteristics of targeted nanoparticles has not been investigated. Antigen expression levels vary greatly across patient tumors and often have heterogeneous expression within tumors. Antigen expression level will affect the ability of targeted nanoparticles to maximize their activity, with implications for indication and patient selection.

HER2/ERBB2, a member of the EGF receptor (EGFR) family of growth factor receptors, is a popular antigen for molecularly targeted therapies. It is highly overexpressed in about 20% of human breast cancers (13) and to a lesser degree in many other cancer types including gastric, lung, bladder, and endometrial (14). HER2 expression levels vary across indications, across patients within an indication, and within individual tumors themselves. Clinically, HER2 protein expression is assessed using HercepTest (Dako), or Pathway (Ventana Medical Systems), semi-quantitative immunohistochemical (IHC) assays that score samples as 0, 1, 2, or 3+, with 3+ reflecting the highest intensity and uniformity of staining. When examined using more quantitative methods, studies in cell lines have mapped 0, 1+, 2+, and 3+ to approximate cell surface receptors numbers of $2 \times 10^5$, $1 \times 10^5$, $5 \times 10^5$ and well over $10^6$ per cell (15). The fraction of cells within a tumor considered to be HER2-positive can also vary from nearly 0% to 100% depending on the patient sample and tumor type (16, 17).

The clinical development of HER2-targeted agents relies on the ability to understand the relationship between HER2 expression level and activity of that particular agent. Trastuzumab, a monoclonal antibody therapeutic targeting HER2, is approved in HER2 “3+” tumors.
and a portion of "2+" tumors that also show gene amplification, as defined by IHC and FISH testing. There are also recent efforts toward studying the performance of trastuzumab in HER2-low breast cancer tumors that are not gene amplified (18, 19). Gastric cancer, another approved indication for trastuzumab, is known to have particularly heterogeneous expression of HER2 (16). In gastric cancer, a distinct HER2-scoring criteria was created, which specifically lowered the fraction of cells that need to exhibit intense HER2 staining to be eligible for HER2-targeted therapies (20). Collectively, this underscores the importance of understanding HER2 levels and heterogeneity in defining patient populations for HER2-directed therapies. A quantitative understanding of the relationship between antigen expression and activity is highly desirable for optimizing the clinical development of molecularly targeted agents.

One HER2-targeted agent currently in clinical development is HER2-targeted Pegylated liposomal doxorubicin (HER2-tPLD; ref. 21). HER2-tPLD is designed to deliver doxorubicin directly to HER2-overexpressing cancers and avoid uptake into nontarget tissues, such as the heart, which express low levels of HER2. HER2-tPLD contains approximately 45 copies of mammalian-derived F5-scFv (anti-HER2) per liposome (22). The F5-scFv was selected for its ability to internalize while not inhibiting HER2 signaling (23). Doxorubicin, the active chemotherapeutic agent, is a backbone of cancer therapy in many HER2 signaling (23). Doxorubicin, the active chemotherapeutic agent, is a backbone of cancer therapy in many HER2 signaling (23).

We and others have shown that targeting HER2-overexpressing cells with HER2-tPLD results in superior activity relative to PLD in preclinical models expressing high levels of HER2 (22, 28, 29). Correspondingly, the absence of a targeting effect has been shown in HER2-negative models where activity was similar to PLD (29). However, the mechanism of action of HER2-tPLD has not been quantitatively studied across the intermediate ranges of HER2 expression. To understand the cellular characteristics of patient tumors that might benefit, and better understand interactions with nontarget tissues, we sought to understand the quantitative relationship between HER2 expression level, cellular uptake, and nuclear delivery of doxorubicin.

Materials and Methods

Liposome preparation

Liposomes were prepared and loaded with doxorubicin as previously described (26, 29). Briefly, the lipid components were hydrogenated soy phosphatidylcholine (HSPC), cholesterol, and PEG-DSPE (3:2:0.3, mol:mol:mol). The anti-ErbB2 (F5)-PEG-DSPE conjugate was prepared and inserted into the liposome as described previously (30, 31) to create HER2-tPLD. Liposomes were also prepared to contain a far red-fluorescent carbocyanine tracer DilC18(5)-DS (D12730—abbreviated DiI5; Life Technologies), which intercalates into the lipid bilayer of the liposome during the extrusion process. The DiI5-labeled liposomes, HER2-tPLD-DiI5 and PLD-DiI5, were prepared as above with DiI5 dye solubilized with the lipid components at 0.3 mol% of total phospholipid. Free doxorubicin was removed using a Sephadex G-75 size exclusion column eluted with HEPES-buffered saline (pH 6.5). The corresponding liposomes without doxorubicin were prepared as well.

Cell culture

AdRr, AU565, Calu-3, HCC202, HCC1569, HCC1954, HeLa, IGROV1, JIMT-1, MCF7, MDA-MB-361, MDA-MB-453, MKN-7, MKN-45, NCI-N87, OE19, SkBr3, SKOV3, U251, UACC812, ZR75-1, ZR75-30, and 4T1 cells were obtained from American Type Culture Collection. OVCAR8 cells were obtained from the National Cancer Institute (NCI) cell repository. Cells were cultured according to manufacturer’s recommendations. BT474-M3 cells were a gift from Hermes Biosciences. MCF-7 clone 18 cells were a gift from Dr. Christopher Benz (Buck Institute, Novato, CA). AdRr-Her2 and OVCAR8-Her2 are stable HER2-overexpressing cell lines derived from AdRr and OVCAR8 cells and were generated in-house. No further cell line authentication was carried out.

Generation of HER2 clones

HER2-expressing clones of 4T1 and HeLa cells were generated as follows: 4T1 and HeLa cells were transfected with a neomycin-selectable expression vector encoding human HER2 (Z2866; GeneCopeia) using transfection reagent MegaTran 1.0 (OriGene) according to the manufacturer’s instructions. Transfected cells were selected in media containing 400 to 500 μg/mL Geneticin/neomycin (Life Technologies) to eliminate nontransfected cells. Surviving cells were expanded under reduced Geneticin concentrations and sorted via flow cytometry to obtain enriched cell populations with HER2 expression exceeding those observed in parental cell lines. The sort-enriched cells were then subcloned by limited dilutions and colonies ranked by HER2 surface levels to obtain representative populations that express different ranges of HER2.
Cell line HER2 quantification

Cells were characterized for human HER2 expression by quantitative fluorescence-activated cell sorting (qFACS) using an anti-HER2 antibody (#340552; BD Biosciences). Fluorescent intensity of HER2 surface staining by flow cytometry was compared with staining with the same antibody of Quantum Simply Cellular microspheres (#815; Bangs Laboratories) to calculate the number of HER2 surface receptors per cell according to the manufacturer’s instructions.

Total cell doxorubicin quantification by HPLC

Each cell line was plated at 25,000 cells per well and incubated at 37°C for 24 hours. Cells were incubated with 15 μg/mL of HER2-tPLD or PLD for 2 hours. The cells were washed twice with PBS, solubilized in 1% acetic acid in methanol at –80°C for 1 hour, centrifuged to remove debris and total doxorubicin quantified using high-performance liquid chromatography (HPLC), as described previously (22).

Total cell doxorubicin measurement by flow cytometry

Adherent cells were incubated with 5 μg/mL of HER2-tPLD for 3 hours followed by washing and incubation in culture media for 21 hours to allow internalization and unpacking of doxorubicin. Cells were then harvested and total uptake was assessed using flow cytometry by measuring the inherent fluorescence of doxorubicin. All flow cytometry data analysis was done with FlowJo v7.2.5 (Tree Star, Inc.).

Quantification of nuclear doxorubicin by high-content analysis

Cells were fixed using 3.7% formaldehyde and washed with PBS-T. Cells were stained with a 1:10,000 dilution of Hoechst 33342 and 1:1,000 dilution of Whole Cell Dye (Pierce) for 30 minutes at room temperature to allow visualization of DNA and the whole cell, respectively. Plates were scanned using the Applied Precision Instruments ArrayWorx High Content Scanner with a 10× objective for Hoechst 33342/whole cell stain (460 nm), doxorubicin (595 nm), and APC/DiI5 (657 nm). Images were analyzed using the software ImageRail (32). An intensity threshold was established for nuclear and whole cell signals. This threshold was applied to all images and used to segment individual cells. Data are presented as the mean pixel intensity for all cells in a given well for the indicated channel.

Liposome internalization

Cell lines were grown and trypsinized using versene:0.05% trypsin in a 1:1 ratio. Cells were pelleted and resuspended in 10 μL ice-cold medium. One milliliter was set aside as an untreated sample. DiI5-labeled HER2-tPLD (0.1 μg/mL) was loaded on cells on ice for 50 to 55 minutes with mixing. Cells were pelleted and washed with 1× 10 mL ice-cold PBS with 1% FBS (FACS buffer) and resuspended in 900 μL ice-cold medium. A 100 μL sample was taken as the t = 0 time point. Eight milliliter of prewarmed RPMI–10%FBS culture media was added to each tube and incubated at 37°C for up to 2 hours. At each time point, 1 mL of cell suspension was removed and added to 9 mL ice-cold FACS buffer on ice.

Cells were pelleted and fixed with 100 μL cytofix solution (#554655; Becton Dickinson) at 4°C. All samples were stained with an anti-PEG antibody (PEG-B-47 rabbit mAb 2061-1; Epitomis) at 1:400 dilution for 30 minutes at 4°C. A goat-anti-rabbit Fab secondary detection reagent conjugated to Alexa Fluor 488 (#4412; Cell Signaling Technology) was used at 1:400 dilution for 30 minutes at 4°C. Cells were washed 3× with 200 μL of FACS buffer between incubations. Samples were resuspended in FACS buffer and data were acquired via flow cytometry.

Internalization rates were estimated by assuming first-order internalization rates and fitting data with a single exponential.

Kinetic computational model

The model is based on mass-action kinetics, except where indicated, and is composed of three components: a HER2 receptor trafficking model, a doxorubicin cellular transport model, and a multivalent HER2-binding model. All parameter values and their sources are listed in Supplementary Table S1.

HER2 trafficking. HER2 trafficking is modeled on the basis of previous work (33). HER2 is synthesized at constant rate k_{syn} and inserted into the cell surface membrane. A fraction of free surface HER2 are internalized and degraded at a net rate k_{deg} (the remaining fraction is internalized and recycled; not explicitly represented).

Doxorubicin cellular transport. Doxorubicin cellular transport is modeled on the basis of published work (34, 35). Free doxorubicin partitions into the outer leaflet of the plasma membrane, characterized by association rate constant k_{f,ol} cell surface area (based on cell radius, assuming spherical cells), and a partition coefficient. Transport from the outer to inner leaflet of the membrane, and back, is mediated by flippases characterized by rate constants k_{flip_in} and k_{flip_out}, respectively. Dissociation from the membrane into the cytosol is similarly characterized by rate constant k_{e,ol}. In the cytosol, doxorubicin reversibly binds to DNA, with association rate k_{f, DNA} and dissociation constant K_{D, DNA}.

Multivalent HER2 binding. The binding of HER2-tPLD is modeled as a semirigid multivalent particle interacting with a surface. HER2-tPLD reacts with a single HER2 receptor (the reversible interaction of a single scFv with a single HER2), with association rate k_{f, LR} and dissociation constant K_{D, LR}. The singly bound HER2-tPLD may then cross-link additional receptors. Initial binding enhances the diffusive rate of encounter with additional HER2 by factor α. Each individual receptor-scFv interaction is characterized by dissociation constant K_{D, LR} (28). Because of the large size of liposomes (100 nm in diameter), total binding is limited by physical space on
the cell surface. The total liposome projected area is calculated and it is used to drive the association rate to zero as the surface becomes fully occupied: \( k_t = k_{0,0} \times [1 - (\text{total liposome projected area})/(\text{cell surface area})] \), where \( k_{0,0} \) is the association rate in the absence of any steric limitations (36). It is also assumed that liposomes cannot freely access the portion of the cell surface that is adherent to the tissue culture plastic—roughly 50%.

The (HER2-tPLD):HER2, \((n \text{ may represent 1 or more HER2 receptors})\) complex is subsequently internalized via receptor-mediated endocytosis at rate \( k_e \). Because HER2-tPLD internalization rates (see Fig. 3) were similar to that of HER2 (33, 37, 38), it was assumed that binding to and cross-linking of HER2 had no effect on internalization rate. Once inside the cell, doxorubicin is released from HER2-tPLD with first-order kinetics, characterized by rate \( k_{\text{recycle,HER2}} \) and stoichiometry of 20,000 doxorubicin molecules per liposome. In the external medium, doxorubicin release occurs much more slowly, at rate \( k_{\text{deg,medium}} \). Once released, the doxorubicin behaves as described in the doxorubicin cellular transport model.

Internalized HER2 that are bound by HER2-tPLD are either degraded at rate \( k_{\text{deg,HER2,lipo}} \) or recycled back to the surface at rate \( k_{\text{recycle,HER2}} \). Internalized liposomes are exocytosed from the cell at rate \( k_{\text{deg,lipo}} \). Nonspecific uptake of liposomes is also represented in the model (33) as a convective transport process, as in fluid-phase uptake, characterized by rate \( k_{\text{fluidphase}} \).

Variation of scFv per liposome is simulated by creating multiple models with different maximum numbers of cross-linking events. The number of cross-linking events can be related to the interaction distance between a liposome and the cell surface, assuming uniform distribution of anti-HER2–binding moieties. This determines the fraction of the liposome surface that can engage in binding events and can be related to the total number of scFv per liposome.

**Implementation.** The model was implemented in MATLAB SimBiology R2011b (The Mathworks). The complete model description is available in Supplementary Materials both in a tabular descriptive. xls format as well as an SBML model file.

**Results**

**Binding and uptake**

To define the levels of HER2 necessary for uptake of HER2-tPLD into cells, HeLa and 4T1 (a murine cell line) were stably transfected to express human HER2. Although the parental 4T1 cell line expresses murine HER2, HER2-tPLD does not bind the murine receptor. Parental HeLa cells expressed approximately \( 2 \times 10^5 \) HER2 per cell. Human HER2-transfected cells were sorted and subcloned to obtain cell populations that express different ranges of human HER2, shown in Fig. 1A and B.

To quantify uptake into the different cell populations, HER2-targeted empty liposomes were with the fluorescent dye, DiI5. Empty liposomes were used as doxorubicin has been reported to cause activation of the cytomegalovirus (CMV) promoter used in the HER2 construct (39, 40). Each HeLa or 4T1-HER2 cell population was incubated with fluorescently labeled targeted liposomes for 3 hours, washed, incubated in fresh media for an additional 21 hours, and then quantified via flow cytometry. Shown in Fig. 1C, five distinct HER2 clones derived from HeLa cells were used to construct a curve of liposome uptake as a function of mean surface HER2 expression level. It was observed that cell-associated DiI5 levels increased with increasing HER2 levels. In Fig. 1D, the HER2 clones derived from 4T1 cells were used to construct a curve of uptake as a function of surface HER2 expression. In this case, subpopulations of each of clone were used to define the relationship between HER2 and uptake. In both cases, there was a sharp increase in targeted liposome uptake at roughly \( 2 \times 10^5 \) HER2 per cell.

Experiments were also carried out to determine if the HER2 threshold for HER2-tPLD uptake would hold across a panel of cell lines. Mean HER2 expression was assessed for cell lines using qFACS. Each cell line was incubated with HER2-tPLD, PLD, or free doxorubicin and total cellular doxorubicin quantified using HPLC. Figure 1E shows the uptake of HER2-tPLD as a function of mean HER2 levels, whereas Fig. 1F shows the uptake of PLD. For select cell lines (4T1 12W7), more than \( 100 \times \) increase in targeted liposome uptake was observed. Increasing levels of HER2 are associated with significant increases in total cell association of HER2-tPLD. Conversely, the uptake of PLD was low and did not correlate with HER2 expression (Fig. 1F). These results suggest that HER2-tPLD is selectively taken up into cells that express HER2, especially in cells which express more than approximately \( 2 \times 10^5 \) receptors per cell. Below \( 2 \times 10^5 \) receptors per cell, the uptake of HER2-tPLD is comparable with levels obtained with PLD.

**Internalization**

The kinetics of association of HER2-tPLD with HER2-expressing cells has been previously shown (22). We next sought to quantify the internalization of HER2-tPLD. Cells were incubated at 4°C to halt endocytosis and then incubated with DiI5-labeled HER2-tPLD to load the surface with bound HER2-tPLD. Cells were washed and transferred to 37°C to stimulate endocytosis. The amount of total cell-associated liposomes was measured by flow cytometry by quantifying changes in the DiI5 mean fluorescence intensity (MFI; Fig. 2A). Simultaneously, changes in surface-bound liposomes were quantified over time using an anti-PEG assay and are shown in Fig. 2B. The total cellular liposome content, measured by DiI5 MFI remains constant, whereas the surface PEG can be shown to decrease over time, indicating internalization. Shown in Fig. 2C is the kinetics of HER2-tPLD internalization for multiple cell lines showing cell-specific internalization rates. Internalization rates were estimated using early time data (0–30 minutes) to avoid confounding effects of recycling (Fig. 2D) and ranged from 0.008 to...
Figure 1. A, HeLa cells were transfected with HER2 to generate multiple clones expressing different levels of HER2. HER2 levels were separately quantified using flow cytometry and calibrated bead standards as described in Materials and Methods. Average values for HER2 per cell are shown in the bottom right of each panel. Cells were incubated with 5 μg/mL of DiI5-labeled HER2-targeted liposomes followed by incubation for 21 hours in cell culture media. Cells were stained with a fluorescently labeled anti-HER2 antibody to enable quantitation of HER2 expression. Each panel indicates the extent of DiI5 signal in individual cells as a function of their relative HER2 expression. B, in a similar fashion as in A, a series of 4T1 HER2-expressing clones were generated and characterized. Each panel indicates the extent of DiI5 signal in individual cells as a function of HER2 expression. C, the mean DiI5 signal from each panel in A is plotted as a function of the mean HER2 level in each HeLa HER2 clone. The circle, square, upward triangle, downward triangle, and diamond refer to clones P7W4, P4W10, P9W4, P9W3, and P6W3, respectively. D, subpopulations of HER2 expression from within each panel in B were used generate the relationship between DiI5 signal and HER2 expression with each 4T1 HER2 clone. Rather than average all the cells within a clone, as done in C, each 4T1 HER2 clone was analyzed as multiple subpopulations by setting up gating for different ranges of HER2 expression. Circles, squares, and triangles refer to subpopulations of clones P11W10, P8W2, and P12W7, respectively. E, various cell lines were incubated with 15 μg/mL of HER2-tPLD for 3 hours. Following washing, total cell-associated doxorubicin was measured by HPLC. Total cell-associated doxorubicin levels are shown as a function of surface HER2 expression levels as characterized using quantitative flow cytometry. The corresponding right y-axis shows the approximate number of liposomes per cell assuming 20,000 doxorubicin molecules per liposome. F, in a similar fashion as in E, cells were treated with PLD and total cell-associated doxorubicin quantified and shown as a function of surface HER2 expression.
0.045 1/min, consistent with published HER2 internalization rates (33, 37, 38).

Nuclear doxorubicin

Nuclear doxorubicin was visualized with microscopy, as shown in Fig. 3A for a low and high HER2-expressing cell line (MCF-7 and ZR-75-30, respectively). Following internalization, the corresponding time course of unpacking and delivery of doxorubicin to the cell nucleus was measured. Multiple cell lines, with varying HER2 expression levels, were incubated with 1 μg/mL HER2-tPLD for up to 8 hours, and nuclear doxorubicin assayed using high-content microscopy. Figure 3B illustrates nuclear doxorubicin accumulation, with increasing accumulation generally corresponding to increasing HER2 expression. MCF7, a cell line expressing relatively few HER2 per cell (1.5 × 10^4) did not show any appreciable uptake. Interestingly, the JIMT-1 cell line (a trastuzumab-resistant cell line; ref. 41) showed little to no nuclear doxorubicin, despite having comparatively high HER2 expression. The amount of nuclear doxorubicin as a function of internalization rate constant (from Fig. 2D) is shown in Fig. 3C. There seems to be a weakly positive relationship between internalization rate and the delivery of doxorubicin to the cell nucleus.

We next evaluated the relationship between cell surface HER2 levels and nuclear doxorubicin levels in 20 cell lines with varied HER2 expression. Cells were incubated with 1.45 μg/mL HER2-tPLD for 24 hours and nuclear doxorubicin quantified using high-content microscopy, shown in Fig. 3D, showing a sharp increase in nuclear doxorubicin starting at roughly 2 × 10^4 HER2 per cell. High HER2 expression was necessary, but not sufficient, for efficient nuclear delivery of doxorubicin, possibly reflecting the activity of drug efflux transporters or other sequestration mechanisms (42). A parallel experiment
Figure 3. A, MCF-7 cells (low-HER2) and ZR-75-30 cells (high HER2) were incubated with 1 μg/mL of HER2-tPLD for 24 hours, as an example of nuclear doxorubicin (Dox) quantification. Cell nuclei were identified using Hoechst dye (left) and doxorubicin (right) identified by its inherent fluorescence. The doxorubicin signal that coincided with the nuclear stain was used as a measure of nuclear doxorubicin content. B, the indicated cell lines were incubated with 1 μg/mL of HER2-tPLD for up to 8 hours and nuclear doxorubicin content assayed by high-content microscopy. The experiment was carried out in triplicate with mean values shown. Error bars were omitted for clarity of presentation (SDs were typically 10% of the mean or less). C, nuclear doxorubicin at 2 and 8 hours (from A) is shown as a function of the HER2-tPLD internalization rate constant from Fig. 2D for the indicated cell lines. D, cell lines with varied levels of surface HER2 expression, as determined by quantitative flow cytometry, were incubated with 1.45 μg/mL of either HER2-tPLD or, in E, doxorubicin for 24 hours and nuclear doxorubicin quantified using high-content microscopy. The cell lines used were: AdrR, AU565, BT-474-M3, Calu3, HCC202, HCC1954, HeLa, HeLa-9W11, JIMT-1, MCF7, MCF7-clone18, MDA-MB-361, MDA-MB-453, NCI-N87, OE19, OVCAR8, OVCAR8-HER2, SkBr3, SKOV3, U251, and ZR75-30.
using free doxorubicin showed no relationship between HER2 expression and nuclear doxorubicin uptake, shown in Fig. 3E.

**HER2-tPLD cellular uptake model**

We constructed a kinetic computational model to solidify our understanding of the various kinetic steps from HER2-tPLD binding (including its dependence on HER2 expression level), internalization, doxorubicin release, and nuclear binding, described in Materials and Methods and shown in Fig. 4A and B.

**Model training.** We incorporated model parameters available from the literature, however, several HER2-tPLD and cell-specific parameters required estimation. A publication on a highly related form of HER2-tPLD (differing only in the antibody used) from Kirpotin and colleagues (43) contains a rich dataset on the effect of varying antibody density and time courses of surface-bound versus internalized liposomes. Using these data, and accounting for the different affinity of the 4D5 scFv [22 nmol/L (44) versus 160 nmol/L for F5 (28)], we were able to generate estimates for the liposome-cell association rate ($k_{r_{LR}}$), the maximum number of cross-linking events ($n$), the cross-linking enhancement factor ($\alpha$), the recycling and degradation rate of internalized HER2 following HER2-tPLD binding ($k_{r_{HER2_tPLD}}, k_{deg_{HER2_tPLD}}$), the liposome/lipid expulsion rate ($k_{deg_{lipopo}}$), the cell-specific HER2 internalization rate ($k_i$), and the rate of nonspecific uptake ($k_{r_{bulkphase}}$). Shown in Fig. 4C is the simultaneous fit of the HER2-tPLD cellular trafficking model to the data from Kirpotin and colleagues (43). Parameter values are listed in Supplementary Table S1. Among the parameters is the estimation that the maximum number of cross-linking (F5-HER2 binding) events is four per liposome-cell interaction. This indicates that four F5-scFv, or roughly 10% of liposome surface, interacts with the cell in cases in which HER2 is not limiting. The value is also consistent with experimental observations, that it is not possible to strip HER2-tPLD off of high HER2-expressing cells with low pH solutions, as one might do with immunoglobulin G (IgG) molecules (data not shown).

**Effect of HER2 expression level on nuclear doxorubicin delivery.** We used the model to simulate the relationship between nuclear doxorubicin and HER2 expression level following incubation with 0.1 nmol/L of HER2-tPLD, shown in Fig. 5A. The model predicts a threshold of uptake of approximately $2 \times 10^5$ HER2 per cell, highly consistent with the experimental data from Figs. 1 and 3. Also, indicated are confidence intervals derived from Monte Carlo simulations assuming up to 3-fold variability in the values of the estimated parameters, illustrating the robustness of this result. At low HER2 expression, binding is inefficient and the small amount of nuclear doxorubicin content arises from two components: nonspecific liposome uptake and external doxorubicin release. In Fig. 5B, we simulate the effect of different numbers of cross-linking events on the delivery of doxorubicin to the nucleus. This indicates that multiple receptor interactions enhance binding and delivery, although there are diminishing returns after two cross-linking events. Finally, at $2 \times 10^5$ HER2 per cell, there would, on average, only be two HER2 under the projected area of each liposome. This is consistent with the beginning of an avidity effect and subsequent threshold-like behavior for binding and uptake.

**Effect of HER2 internalization rate on nuclear doxorubicin delivery.** Because of the interest in antigen selection for the development of targeted nanoparticles, we also explored the importance of internalization rates. Shown in Fig. 6A, we simulated the kinetics of nuclear doxorubicin delivery for a range of internalization rates, based on Fig. 2D. Here, we observed that, within the physiologic range of internalization rates for HER2, there is a weakly positive relationship between internalization rate and nuclear doxorubicin delivery. This result was confirmed by experimental observations that internalization rates were modestly correlated with increased nuclear doxorubicin (see Fig. 3C). A sensitivity analysis, shown in Fig. 6B, further indicates that the rate of internalization plays a comparatively small role relative to other kinetic steps. HER2 expression level is the most important factor, followed by two liposome-specific parameters: the scFv affinity ($k_{r_{IL}}$) and the cross-linking enhancement factor ($\alpha$). Internalization is a necessary step for effective delivery, but it is not predicted to be kinetically limiting.

**Discussion**

In this work, we examined the quantitative relationship between HER2 expression levels and the performance of HER2-tPLD. We show that HER2-tPLD shows increased cellular uptake with increasing HER2 expression and corresponding increases in delivery of doxorubicin to the nucleus. Most notably, we found a threshold effect, taking place at approximately $2 \times 10^5$ HER2 per cell, at which there was enhanced binding and uptake of HER2-tPLD relative to PLD. This threshold held both within a single cell background and across multiple cell lines. This suggests that a critical density of HER2 receptors is necessary on the cell surface for efficient binding of HER2-tPLD and cross-linking of receptors. The presence of a threshold effect is further shown using a computational model that captures the key steps in HER2-tPLD-mediated delivery of doxorubicin. Using the model, we are able to show that the threshold effect arises from multiple low-affinity interactions leading to high avidity, estimate the extent of cross-linking of HER2, and characterize the role of internalization on nuclear doxorubicin delivery.

Experimentally, we found variability across cell lines at all levels. Internalization of HER2-tPLD varied considerably, but was within ranges previously observed. Differences in internalization rate may reflect different extents of heterodimerization with EGFR; increased heterodimerization with EGFR might result in slightly increased internalization of HER2-tPLD (33). There was a weak correlation between internalization rate and nuclear doxorubicin accumulation confirmed via simulation.
Figure 4. A, the surface binding of HER2-tPLD is proposed to occur via a multistep mechanism. HER2-tPLD associates with the cell surface and initially binds a single HER2 receptor, characterized by the affinity of the F5 scFv for HER2 ($K_{DLR}$), and an off-rate ($k_r$). On-rate, $k_{f, L} = k_r/K_{DLR}$. Subsequent cross-linking of additional receptors in the two-dimensional plane of the membrane occurs at an enhanced rate ($u/k_{f, L}^2$), where $u$ is a cross-linking enhancement factor. The off-rate from $n$-bound HER2 to $(n-1)$-bound HER2 is $n \times k_r$ to account for the number of possible unlinking events that can take place. The total number of cross-linking events possible is estimated on the basis of experimental data. B, the proposed mechanism for the cellular trafficking of HER2, doxorubicin, and HER2-tPLD is shown. Before HER2-tPLD binding, HER2 levels are assumed to be at steady state, characterized by synthesis rate $k_{syn}$ and degradation rate $k_{deg,HER2}$. At steady state, basal HER2 internalization and recycling are balanced and therefore, not represented. Following surface binding to one or more HER2, as described in A, HER2-tPLD is internalized at rate $k_{e,HER2}$. HER2-tPLD may also be taken up via a nonspecific fluid-phase uptake mechanism characterized by rate $k_{e, fluidphase}$. Inside the cell, doxorubicin is released from HER2-tPLD with first-order kinetics at rate $k_{rel,cell}$. Intact and/or empty liposomes are expelled from the cell at rate $k_{deg, lipo}$. HER2 that has been internalized with HER2-tPLD may either be recycled to the cell surface at rate $k_{x,HER2-lipo}$ or degraded at rate $k_{deg,HER2-lipo}$. Free doxorubicin within the cell can bind to target DNA, characterized by association rate constant $K_{a, DNA}$ and off-rate $k_{r, DNA}$. (Continued on the following page.)
Effect of HER2 Level on HER2-Targeted Liposome Drug Delivery

Figure 5. A, using the kinetic model, nuclear doxorubicin at 24 hours was simulated following stimulation with 1 μg/mL of either HER2-tPLD (dotted line with open circles) or PLD (solid line with filled circles) for varying levels of HER2 expression. The robustness of the threshold effect to variations in estimated parameters was shown by conducting 50 Monte Carlo simulations, assuming up to a 3-fold range in value for each estimated parameter. The regions indicated by shading or dashed lines indicate the mean simulation result plus or minus one SD. B, the role of HER2 cross-linking by HER2-tPLD on the uptake threshold curve from A was investigated by computationally varying the maximum number of cross-linking events that could take place. Simulation results corresponding to 0, 1, 2, and 3 cross-linking events are shown with the following line styles, respectively: solid line with circles, solid, dashed, dotted, and dash-dotted lines.

results (Fig. 6), which suggests this plays a comparatively small role in overall delivery. Total delivery, in general, increased with HER2 expression. One interesting exception was the JIMT-1 cell line, a cell line found to have diminished capacity for trastuzumab binding due to elevated MUC4 expression (41). Our data indicate that there was also reduced nuclear doxorubicin delivery via HER2-tPLD in JIMT-1. This suggests that similar mechanisms may affect binding ability. Cell surface clustering of HER2 may further be responsible for the apparent discordance between receptor levels and binding/uptake of HER2-tPLD.

The computational model was built upon previously validated models for HER2 trafficking and doxorubicin cellular transport, trained on data from a single cell line and it correctly predicted the threshold of HER2 uptake observed experimentally across multiple cell lines. The model does not prove that the proposed binding and trafficking mechanisms are correct, but simply shows that they are consistent with observed experimental data. In the interest of simplicity, the model does have some limitations. The model does not explicitly represent the dimerization state of HER2 with other ErbB family receptors. These interactions could conceivably alter binding through masking of epitopes and/or affect internalization rates of HER2-bound liposomes. The clear trend in uptake as a function of HER2 across multiple cell lines suggests that these effects are comparatively minor; however, they might explain cell line-specific deviations and could implicitly be represented by altered binding and/or HER2 internalization rate constants. The model also assumes a uniform distribution of HER2 on the cell surface. Receptor clustering would result in locally higher binding up to a point at which liposomes would sterically interfere with binding of additional liposomes. This could potentially lower the receptor threshold for effective binding and uptake on portions of the cell surface although it may result in lowered total cell binding.

Other small discrepancies between the model and training data also suggest possible opportunities for refinement. For example, the predicted kinetics of nuclear doxorubicin accumulation was slightly slower in the simulation than experimentally observed. On the basis of a separate analysis (not shown), the most likely explanation for this is that we have underestimated the rate of doxorubicin release from the liposome within the cell subsequent to internalization and/or the rate of liposome–cell association. Finally, the use of the computational approach could also enable optimization of immunoliposome design criteria such as the number of antibodies and antibody affinity. Modeling liposomes with different size would, in principle, follow a similar

(Continued.) Doxorubicin may partition into the inner leaflet of the cell membrane characterized by mass transfer coefficient $k_{e,UL}$ and a partition coefficient. Transport from the inner to outer leaflet (and back) by flipases characterized by rate $k_{flipase_{out}}$ (or $k_{flipase_{in}}$). Dissociation from the outer leaflet of the membrane into the medium is analogously characterized by mass transfer coefficient $k_{e,UL}$ and the same partition coefficient. Finally, doxorubicin may be released from HER2-tPLD in the medium, assuming first-order kinetics, at rate $k_{e,medium}$, C, the kinetic model described in A and B was trained on experimental data from Kirpotin and colleagues (43). Experimental data are shown as black circles and model simulations as solid lines. The data from Kirpotin and colleagues were measured in SKBr3 cells (expressing ~1.6 x 10^6 HER2/cell) using a similar HER2-targeted liposome containing doxorubicin, however, 4DS Fab was used as a targeting antibody rather than F5. Left top and bottom, surface-bound and internalized phospholipid (PL) as a function of time, respectively, for a liposome with 78 Fab/liposome. Total associated phospholipid (top middle and top right) and the percentage internalized (bottom middle and bottom right) were measured as a function of different Fab/liposome at either 2 hours (middle) or 6 hours (right). The model was fit to all data simultaneously, adjusting for the difference in affinity for HER2 between 4DS and F5. The following parameters were estimated from the data: $k_{e,UL}$, $k_{e,Hk}$, $k_{flipase_{out}}$, $k_{flipase_{in}}$, $k_{e,HER2}$, $k_{e,medium}$, $k_{e,HER2}$, $k_{e,HER2}$, and $k_{flipase_{out}}$, with their values shown in Supplementary Table S1.

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framework. However, changes in liposome size would affect the surface density of antibody and might alter the efficiency of cross-linking.

Targeted nanoparticle-mediated delivery of doxorubicin can greatly increase delivery in vitro relative to untargeted liposomes. Free doxorubicin, however, is still the fastest manner in which to deliver doxorubicin to the nucleus in vitro, as indicated by the higher magnitudes of overall nuclear doxorubicin signal in Fig. 3C compared with 3B. Nevertheless, free doxorubicin has disadvantages in that nontarget tissues, such as the heart, are also exposed. Nuclear delivery of doxorubicin via HER2-tPLD is necessarily slower than free drug due to steps of binding, internalization, and release of drug. The extended circulation of liposomes in vivo relative to conventional doxorubicin overcomes the relatively small differences in delivery at the cellular level and can further contribute to increased overall tumor exposure relative to free drug. It remains to be shown that the threshold effect observed in this work holds in vivo. Evaluation of a threshold across models of varying HER2 expression (both in vitro and in vivo) is potentially confounded by variable and heterogeneous delivery in vivo as well as variable inherent sensitivity to doxorubicin. However, exploiting heterogeneity of HER2 expression within a suitably tumorigenic xenograft model may enable demonstration of the threshold for delivery effects.

Further work is necessary to bridge from nuclear doxorubicin binding to cell killing, taking into account cellular factors that affect individual responses to doxorubicin including innate and adaptive signaling mechanisms contributing to resistance. This would be shown by cell lines that take up significant nuclear doxorubicin but do not show appreciable cell death. Although there are no approaches in clinical use to address this aspect, this is an area that has received much attention. The association between TOP2A or TIMP-1 amplification with response to anthracyclines is one such avenue that has been pursued (45, 46). In addition, the stimulation of ErbB3-mediated survival signaling in response to doxorubicin has been observed in some ovarian cancer cell lines (47). Sequestration of doxorubicin by lysosomal proteins possibly contributing to treatment resistance in breast cancer has been reported as well (48). Maximizing drug delivery combined with approaches to combat innate or adaptive mechanisms of cell resistance currently seem promising.

It is also important to consider antigen expression levels in nontarget tissues. For example, HER2 is expressed at low levels in heart tissue (49). Our cell line and modeling work herein support earlier data showing that HER2-tPLD does not bind and enter human cardiomyocytes, which express low levels of HER2 (22). In addition, liposomal encapsulation also prevents extravasation into heart tissue in vivo (22). Tuning the number of binding arms and their affinity could enable optimization of the therapeutic index of this class of agents for other antigens. The modeling framework presented could also be leveraged to optimize such design criteria.

Although it is clear that the highest HER2 expression levels are best for optimal HER2-tPLD performance, differential uptake into cells with moderate HER2 expression (2 × 10^5–8 × 10^5 HER2/cell) was also observed. This is due to the use of HER2 for docking and mediating liposome internalization, rather than relying on HER2 amplification or oncogene-addiction for the mechanism of action. It will ultimately be necessary to unravel the relationship(s) between heterogeneity of HER2 expression and responses in vivo. For example, one could determine the threshold of HER2 expression required for antitumor activity, assuming homogeneous expression of HER2 as well as...
To determine the percentage of cells expressing high HER2 levels that are necessary to observe responses to HER2-tPLD. Nonetheless, this work suggests an opportunity to target patient tumors with moderate levels of HER2 expression where few treatment options exist. This could greatly increase the potential patient population beyond that of traditionally defined “HER2-positive” disease. In these cases, it may be especially important to understand the ability to target small metastases and/or identify tumors that show high degrees of overall liposome deposition.

Disclosure of Potential Conflicts of Interest

T.J. Wickham has ownership interest (including patents) in Merrimack Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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References


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Correction: Impact of Tumor HER2/ERBB2 Expression Level on HER2-Targeted Liposomal Doxorubicin-Mediated Drug Delivery: Multiple Low-Affinity Interactions Lead to a Threshold Effect

In this article (Mol Cancer Ther 2013;12:1816–28), which appeared in the September 2013 issue of Molecular Cancer Therapeutics (1), the authors regret that the article title was incorrect.

The authors forgot to mention the company code of HER2-targeted liposomal doxorubicin (MM-302) for reference. The company code is important for other investigators to link this work with other ongoing clinical trials that are using MM-302. The publisher recommended that correcting the title was the most appropriate means for making this link. The correct title is given below.

"Impact of Tumor HER2/ERBB2 Expression Level on HER2-Targeted Liposomal Doxorubicin (MM-302)-Mediated Drug Delivery: Multiple Low-Affinity Interactions Lead to a Threshold Effect".

Reference

Impact of Tumor HER2/ERBB2 Expression Level on HER2-Targeted Liposomal Doxorubicin-Mediated Drug Delivery: Multiple Low-Affinity Interactions Lead to a Threshold Effect
