Bispecific Antibodies and Antibody–Drug Conjugates (ADCs) Bridging HER2 and Prolactin Receptor Improve Efficacy of HER2 ADCs

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Abstract

The properties of cell surface proteins targeted by antibody–drug conjugates (ADCs) have not been fully exploited; of particular importance are the rate of internalization and the route of intracellular trafficking. In this study, we compared the trafficking of HER2, which is the target of the clinically approved ADC ado-trastuzumab emtansine (T-DM1), with that of prolactin receptor (PRLR), another potential target in breast cancer. In contrast to HER2, we found that PRLR is rapidly and constitutively internalized, and traffics efficiently to lysosomes, where it is degraded. The PRLR cytoplasmic domain is necessary to promote rapid internalization and degradation, and when transferred to HER2, enhances HER2 degradation. In accordance with these findings, low levels of cell surface PRLR (≈30,000 surface receptors per cell) are sufficient to mediate effective killing by PRLR ADC, whereas cell killing by HER2 ADC requires higher levels of cell surface HER2 (~10^6 surface receptors per cell). Noncovalently cross-linking HER2 to PRLR at the cell surface, using a bispecific antibody that binds to both receptors, dramatically enhances the degradation of HER2 as well as the cell killing activity of a noncompeting HER2 ADC. Furthermore, in breast cancer cells that coexpress HER2 and PRLR, a HER2xPRLR bispecific ADC kills more effectively than HER2 ADC. These results emphasize that intracellular trafficking of ADC targets is a key property for their activity and, further, that coupling an ADC target to a rapidly internalizing protein may be a useful approach to enhance internalization and cell killing activity of ADCs. Mol Cancer Ther; 16(4): 681–93. ©2017 AACR.

Introduction

Breast cancer remains a disease of high unmet medical need (1). Monoclonal antibodies have proven to be effective therapies for this disease, with two antibodies targeting HER2 (trastuzumab and pertuzumab) approved for treatment.

Recently, antibody–drug conjugates (ADCs) were introduced into clinical practice with the goal of improving efficacy of antibody-based treatments (2, 3). ADCs are designed to selectively kill cancer cells by combining the specificity of a monoclonal antibody with the intracellular delivery of a highly potent cytotoxic agent that usually targets tubulin or DNA. ADC targets need to be selectively expressed on tumor cells versus vital normal cells and must be able to deliver sufficient amounts of ADC to the intended intracellular compartments to ensure efficient ADC processing and drug release (4).

The HER2-directed ADC, ado-trastuzumab emtansine, or T-DM1 (trastuzumab conjugated with a noncleavable linker to tubulin inhibitor, DM1) was approved by the Food and Drug Administration for the treatment of patients with HER2-overexpressing metastatic breast cancer who have received prior treatment with trastuzumab and a taxane (5, 6). The efficacy of T-DM1 has been demonstrated only in patients with HER2 overexpression defined as IHC3+ (7) or FISH amplification ratio ≥ 2, which is only about half of metastatic breast cancer patients with detectable IHC HER2 expression (8, 9). Thus, there is a need for HER2-directed ADCs that are more effective in patients expressing low/moderate levels of HER2. Because targeting tissues with low HER2 expression might also induce unwanted toxicity, using bispecific antibodies (bsAb) to HER2 and a target selectively expressed in breast tumors may overcome this potential limitation.

We have recently generated an ADC against such breast cancer target, prolactin receptor (PRLR), a type 1 cytokine receptor that functions in mammary gland development and lactation (M.P. Kelly; unpublished data). PRLR is expressed in a subset of breast tumors (10, 11) and is implicated in the pathogenesis of breast cancer (12, 13). However, unlike HER2, PRLR is not commonly amplified in breast cancer and is generally expressed at much lower levels on the cell surface than HER2. Despite the lower levels of expression, PRLR ADC (PRLR antibody conjugated with noncleavable linker to DM1) induces efficient target-mediated killing of breast cancer cells.
in vitro and potently inhibits the growth of breast tumor xenografts in vivo (M.P. Kelly; unpublished data).

Because internalized HER2 appears to predominantly recycle to the cell surface rather than traffic to lysosomes (14, 15), we hypothesized that the difference in killing efficiency between PRLR and HER2 ADCs is due to the difference in lysosomal trafficking of their targets. Indeed, we demonstrate that in breast cancer cells, PRLR, but not HER2, constitutively traffics to lysosomes and is rapidly degraded. Consistent with this finding, we show that noncovalently cross-linking HER2 and PRLR at the plasma membrane using HER2xPRLR bsAbs triggers HER2 degradation in lysosomes. Naked HER2xPRLR bsAbs augment killing of breast cancer cells by a noncompeting HER2 ADC. Further, a HER2xPRLR bspecific ADC (bsADC) kills breast cancer cells more effectively than HER2 or PRLR ADCs. Our results suggest that coupling an ADC target to a rapidly internalizing protein may be a useful approach to enhance internalization and cell killing activity of ADCs.

Materials and Methods

Antibodies, ADCs, and other reagents

PRLR antibody, a fully human monoclonal antibody to human PRLR, was generated by immunizing VelocImmune mice (16, 17). HER2 antibody (“in-house trastuzumab”) is a human monoclonal antibody with primary sequence identical to that of trastuzumab (18, 19). PRLR antibody and HER2 antibody were produced in Chinese hamster ovary cells at Regeneron.

PRLR antibody and HER2 antibody were conjugated via surface lysines to the maytansine derivative DM1, using a noncleavable, hetero-bifunctional linker (succinimidyl trans-4-[maleimidylmethyl] cyclohexane-1-carboxylate, SMCC; Supplementary Fig. S1A). The PRLR and HER2 ADCs had average drug-to-antibody ratios (DAR) of 3.4 and 2.4, respectively. An isotype-matched nonbinding control ADC was prepared as described above at average DAR of 3.5.

A bsAb with HER2 and PRLR arms (HER2(T)xPRLR) was generated using the “knobs-into-holes” approach (20). The HER2(T) arm was derived from the primary sequence of trastuzumab. The PRLR arm was derived from a fully human VelocImmune PRLR antibody designated H1H7672P2.

Additional HER2 and PRLR antibodies were generated in VelocImmune mice that express a single (“universal”) light chain (21) to obviate the potential for heavy-light chain mispairing. HER2xPRLR bsAbs were generated as previously described (22) using HER2 antibodies that do not cross-compete with trastuzumab. HER2xPRLR bsADC is composed of HER2xPRLR bsAb1 conjugated to maytansine derivative DM1 via a noncleavable linker as described above with average DAR of 3.3 (see Supplementary Materials and Methods for details).

Recombinant human prolactin hormone (PRL) was from R&D Systems. Cycloheximide (CHX; ref. 23), bafilomycin A1 (BafA1; ref. 24), chloroquine (25), and monensin (26) were from Sigma. Bortezomib (27) and lactacystin (28) were from Selleck Chemicals.

Breast cancer cell lines and transient transfections

All human breast cancer cell lines used in this study were obtained from the American Type Culture Collection, authenticated by short tandem repeat profiling (IDEXX Bioresearch), and cultured under standard conditions. Jump-In T-REx HEK293 cells were from Thermo Fisher Scientific. Transient transfections were performed using pTFl R4 CMV-TO vector. To generate T47D cell line overexpressing HER2, T47D cells were stably transfected with human full-length HER2 (HER2 FL), subconcloned into pBG984 plasmid, and G418 (500 µg/mL) selection was applied. All experiments were conducted with low-passage cell cultures (passage 10).

Inducible expression of recombinant receptors

Lenti-X Tet-On Advanced Inducible Expression System (Clontech) was used to generate HEK293 cells expressing either full-length PRLR (PRLR FL), HER2 FL, or HER2-PRLR chimera constructs in a tetracycline-controlled fashion. HEK293 cells were cotransduced with lentiviruses derived from the pLVX-Tet-On advanced regulator vector and the pLVX-Tight-Pur-GOI response vector with subsequent selection using G418 and puromycin, as suggested by the manufacturer.

Jump-in T-REx HEK293 cells were generated to express either PRLR FL or PRLR truncation mutant lacking the cytoplasmic domain in a tetracycline-controlled fashion. The Jump-In T-REx HEK293 cells containing pre-engineered R4 site and stably expressing the tetracycline repressor protein were transfected with the desired expression construct followed by 14 to 21 days of antibiotic selection. Cell pools were expanded and analyzed for gene expression using RT-PCR.

Recombinant receptor expression was induced by doxycycline (0.7 µg/mL) for 24 hours unless indicated otherwise.

Flow cytometry

Binding of PRLR and HER2 antibodies to wild-type and recombinant HEK293 cells was evaluated by flow cytometry as follows: cells were blocked in PBS/BSA on ice, left unstained, or incubated with PRLR or HER2 antibodies, followed by Alexa Fluor 488–conjugated goat anti-Human IgG Fab Fragment (Jackson ImmunoResearch Laboratories). Alternatively, cells were incubated only with Alexa Fluor 488–conjugated goat anti-Human IgG Fab Fragment (“Secondary Ab only”). Samples were analyzed on the AccuriC6 Cytometer using FlowJo X10.0.07 software. HER2 or PRLR surface levels were expressed as F/B ratio; Fold change in mean fluorescence intensity above Background (“Secondary Ab only”).

For Quantitative Flow Cytometry, PRLR and HER2 antibodies were conjugated to R-Phycoerythin (R-PE) using standard protocols. Quantum R-PE-MESF kit (Bangs Laboratories) was used for the quantitation of PE fluorescence intensity in Molecules of Equivalent Soluble Fluorophore (MESF). All samples were analyzed on the AccuriC6 Cytometer using FlowJo X10.0.07 software. Fluorescence to protein ratio (F/P) was determined for PE-conjugated PRLR and HER2 antibodies using Simply Cellular standard beads (Bangs Laboratories). The number of surface receptors per cell was determined by dividing MESF value at saturating concentration of an antibody by the value of respective F/P ratio.

Direct cell count–based cytotoxicity assay and cell-cycle arrest measurements

Tumor cells were grown in 96-well CellCoat optical plates (Greiner) at 37°C with 5% CO2 overnight. ADCs were added to cells at indicated concentrations in triplicates. For direct cell count–based cytotoxicity assay, cells were incubated with ADC for 3 days (unless indicated otherwise) and then fixed, permeabilized, and nuclei-stained in one step, as described in ref. 29.
Whole well images were acquired on automated microscope ImageXpress Micro (MD) and analyzed for total nuclei count using MetaXpress software (MD). Cell viability was determined as the percentage of Hoechst DNA stain–labeled nuclei for ADC-treated cells relative to untreated cells. IC50 values were calculated using GraphPad Prism software.

Cell-cycle arrest was measured using increase in percentage of early mitotic cells as detected with anti–phospho-Histone H3 (Ser10) antibody, which labels cells undergoing chromosome condensation during mitosis (30). Twenty-four hours following ADC treatment, cells were fixed with 4% PFA, permeabilized with 0.1% Triton X-100, and stained with Alexa Fluor 647–labeled anti–phospho-HistoneH3 (Ser10; CST) antibody solution containing Hoechst33342. Whole well images were acquired and analyzed using MetaXpress software.

Trafficking assays
PRLR or HER2 antibodies were conjugated with the fluorophores CF594 (Biotium) or Alexa Fluor 405 or with pHRedo (Thermo Fisher Scientific) according to the manufacturer’s instructions. Noninternalized primary antibodies on the cell surface were detected with Alexa Fluor 488–conjugated goat anti-human IgG Fab Fragment (Jackson ImmunoResearch Laboratories). Lysosomes were labeled by incubating cells with fluorescein-3 kDa dextrans for 1 hour. Nuclei were stained with Hoechst 33342. Epifluorescence images were acquired on ImageXpress Micro at 40x. Confocal images were acquired on Zeiss LSM780 at 40x. Colocalization was quantified based on Pearson’s r coefficient using ImageJ software.

Surface plasmon resonance
The affinities of PRLR or HER2 antibodies for human PRLR or HER2 were measured using a Biacore T200 (GE Healthcare; see Supplementary Materials and Methods for additional details). Kinetic parameters were obtained by globally fitting the data to a 1:1 binding model using Biacore T200 Evaluation Software. The equilibrium dissociation constant (Kd) was calculated by dividing the dissociation rate constant (ka) by the association rate constant (kb).

Western blotting
CHX was used at 50 μg/mL. Cell lysates (RIPA buffer) were resolved on 4% to 20% Novex Tris-Glycine gels and blotted to PVDF membranes (Novex). The following antibodies were used for primary labeling of membranes: PRLR antibodies (Life Technologies; 35-9200), HER2 antibodies (DAKO; A0485), α-tubulin antibodies (Sigma Aldrich; T5168), β-actin antibodies (Santa Cruz Biotechnology; sc-47778), and GAPDH antibodies (GeneTec; GTX1003131). Labeling with primary antibodies was followed by horseradish peroxidase–conjugated secondary antibody (GE Healthcare) at 1:5,000 and chemiluminescence detection.

Results

PRLR, but not HER2, is constitutively internalized and traffics efficiently to lysosomes

Previous reports indicate that certain class 1 cytokine receptors, including PRLR, undergo rapid internalization (32–34). We reasoned that such receptors might be better ADC targets at low surface expression levels than proteins that do not rapidly and constitutively internalize, such as HER2. To measure PRLR and HER2 internalization, fluorescently labeled PRLR or HER2 antibodies were bound to T47D breast cancer cells at 4°C and allowed to internalize at 37°C for varying times. Internalization of PRLR antibody was evident by the intracellular accumulation of primary antibodies, whereas HER2 antibody remained on the cell surface (Fig. 1A, top and bottom). As shown on the graph in Fig. 1A, about 90% of PRLR antibody was internalized within 60 minutes, whereas minimal internalization was observed for HER2 antibody. The PRLR antibody used in this experiment blocks PRL binding and signaling (10), indicating that PRL present in the medium is not required for internalization.

Upon internalization into early endosomes, antibodies and their targets may follow a recycling pathway leading through recycling endosomes back to the cell surface, and/or a lysosomal pathway leading through late endosomes to lysosomes (35). To evaluate lysosomal trafficking of PRLR and HER2, we measured the kinetics by which dextran-labeled lysosomes were occupied by internalized fluorescently labeled PRLR or HER2 antibodies. Confocal images showed PRLR antibody signal progressively moved away from the plasma membrane into dextran-labeled lysosomes, whereas the HER2 antibody signal remained in the plasma membrane (Fig. 1B, top and bottom). After 70 minutes, PRLR antibody occupied approximately 80% of the lysosomal compartment, whereas HER2 antibody occupied approximately 20% of the lysosomal compartment (graph in Fig. 1B). Upon even longer incubation times (24 hours), PRLR, but not HER2 antibody, accumulated in low pH vesicles, as detected using the pH-sensitive marker pHRedo (ref. 36; Supplementary Fig. S1B). This observation provides further confirmation that PRLR, but not HER2, traffics to the lysosomal compartment.

Low levels of cell surface PRLR, but not HER2, are sufficient to mediate ADC cell killing in vitro

To determine if the dramatic differences in internalization rates and lysosomal trafficking of PRLR and HER2 would translate into differences in ADC-mediated cell killing, we compared the efficacy of PRLR and HER2 ADCs in breast cancer cells expressing high or low surface levels of PRLR and HER2. As shown in Fig. 2A, breast cancer cell line SK-BR-3 expressed high surface levels of HER2 (1.19 × 106 receptors per cell), and very low surface levels of PRLR (1.04 × 104 receptors per cell). As expected, HER2 ADC induced robust killing of SK-BR-3 cells (0.2 nM IC50), whereas PRLR ADC had minimal effect on cell viability. In contrast, the T47D breast cancer cell line expressed a relatively low surface level of HER2 (4.00 × 104 receptors per cell) and an even lower surface level of PRLR (2.73 × 104 receptors per cell; Fig. 2B). Consistent with the rapid internalization and degradation of PRLR, T47D cells were sensitive to PRLR ADC–induced cell killing (0.4 nM IC50), but were completely resistant to HER2 ADC. Importantly, ADCs generated using other PRLR antibodies were similarly effective in T47D cells (not shown).

To assess whether increasing HER2 surface expression in T47D cells would increase their susceptibility to killing by HER2 ADC, we generated a T47D cell line stably overexpressing HER2 (T47D/HER2). As shown in Fig. 2C, similar to the T47D parental cell line, T47D/HER2 cells expressed low PRLR surface levels (3.41 × 104 receptors per cell) and were sensitive to PRLR
Figure 1.
PRLR, but not HER2, undergoes rapid, constitutive lysosomal internalization. Confocal microscopy images of T47D cells showing PRLR or HER2 internalization (A) and lysosomal trafficking (B) were taken at different time points. For each time point, two representative confocal sections (perinuclear and supranuclear) and the XZ view are shown. A, PRLR or HER2 primary antibodies were labeled with CF594 (red); noninternalized primary antibodies on the cell surface were stained with Alexa Fluor 488–conjugated secondary antibodies (green). Scale, 10 μm. A, graph. Internalization of PRLR and HER2 was quantified as the percentage of primary antibody colocalized with secondary antibody at a particular time point (% surface receptor; mean ± SE). B, PRLR or HER2 primary antibodies were labeled with CF594 (red), lysosomes were labeled with fluorescein-3 kDa dextrans (green). Scale, 10 μm. B, graph. Colocalization of PRLR or HER2 with lysosomes over time was quantified as the percentage of 3 kDa dextrans colocalized with receptor antibody (% Lysosomes colocalizing with internalized receptor; mean ± SE).
ADC (0.5 nM IC50). Contrary to the T47D parental cell line, surface levels of HER2 were intermediate in T47D/HER2 cells (2.07 × 105 receptors per cell), and a certain degree of sensitivity to HER2 ADC–mediated killing was restored (40 nM IC50). Taken together, these data confirmed previously published observations that high HER2 surface levels are required to mediate HER2 ADC activity (37). In contrast, and consistent with our intracellular trafficking studies, relatively low surface levels of PRLR are sufficient to mediate robust ADC killing of tumor cells in vitro (IC50 < 1 nM).

PRLR, but not HER2, is rapidly degraded in lysosomes

Multiple studies have shown that antibodies can promote internalization and lysosomal trafficking of their target receptors (38–40). In order to investigate the turnover of PRLR and HER2 in the absence of their respective antibodies, protein turnover in T47D cells was evaluated in a CHX chase assay. As shown in Fig. 3A, inhibition of protein synthesis with CHX had little effect on HER2 levels, whereas PRLR degradation was first evident 60 minutes after CHX addition and was almost complete after 120 minutes. These results indicate that PRLR, but not HER2, undergoes rapid turnover in T47D cells in the absence of antibodies.

To study PRLR turnover in a different cell system, HEK293 cells induced to express PRLR were preincubated with either dimethyl sulfoxide (DMSO) or with the lysosomal inhibitors BafA1, chloroquine, or monensin, and subjected to CHX treatment for different times. Results of this experiment (Fig. 3B) demonstrated that overexpressed PRLR is rapidly degraded in HEK293 cells with kinetics similar to those observed in T47D cells and that lysosomal inhibitors chloroquine and monensin completely abrogated PRLR degradation in the CHX chase assay, whereas another lysosomal inhibitor, BafA1, strongly reduced PRLR degradation. In contrast, the proteasome inhibitors bortezomib and lactacystin had little or no effect on turnover of overexpressed PRLR (Supplementary Fig. S2), suggesting a lack of proteasome involvement. Moreover, PRLR degradation in the CHX chase assay was not significantly affected by addition of PRLR antibody or PRL (Supplementary Fig. S3), further indicating that constitutive PRLR turnover is largely independent of PRL and PRLR antibody.

To evaluate the role of lysosomal processing in PRLR and HER2 ADC–mediated cell-cycle arrest, we treated T47D cells with different concentrations of PRLR or HER2 ADCs in the presence or absence of BafA1. We found that PRLR, but not HER2 ADC, increased the percentage of early mitotic T47D cells in a dose-dependent manner (Fig. 3C). This effect was completely abolished by coincubation with BafA1, indicating that acidic lysosomal pH is needed for PRLR ADC–mediated cell-cycle arrest. Thus, rapid constitutive internalization of cell surface PRLR may allow for efficient accumulation of PRLR ADC in lysosomes, supplying enough catabolized lysine-MCC-DM1 (41) to trigger cell-cycle arrest and cell death.

PRLR turnover is mediated by its cytoplasmic/transmembrane domain and is required for ADC efficiency

To identify domain(s) of PRLR that confer efficient trafficking to lysosomes, receptor chimeras consisting of extracellular (ecto), transmembrane (TM), and cytoplasmic (cyto) domains of PRLR and HER2 were constructed (Fig. 3D).

To study internalization of the receptor chimeras, HEK293 cells expressing either PRLR FL, HER2 FL, HER2ectoPRLRectoHER2cytoTM, or PRLRectoHER2cytoTM were transiently transfected in 293 cells, and subjected to ice-to-37°C internalization assay with either HER2 or PRLR antibodies. As shown in Fig. 3E, overexpressed PRLR FL, but not HER2 FL, completely internalized within 1 hour. Importantly, PRLRectoHER2cytoTM did not internalize, whereas HER2ectoPRL RectoTM completely internalized within 1 hour, indicating that the PRLR transmembrane and cytoplasmic domains are essential for rapid internalization of the receptor.

To demonstrate the importance of the PRLRectoTM in mediating the effect of PRLR ADC on cell-cycle arrest, T-REx HEK293 cells were induced with different doxycycline concentrations to express different surface levels of PRLR, HER2, or PRLR-HER2 chimeras (Fig. 3F). As shown in Fig. 3F, PRLR or HER2 ADC–induced cell-cycle arrest positively correlated with surface levels of the expressed receptors. However, HER2 ADC required at least 10-fold more HER2 on the surface of HEK293 cells
PRLR, but not HER2, is rapidly degraded in lysosomes, which is essential for ADC-mediated cell-cycle arrest. 

A, T47D cells were incubated with CHX for the indicated times, lysed, and processed for Western blotting.

B, HEK293 cells induced to express PRLR FL were pretreated for 2 hours with DMSO or BafA1 (1 μM) or chloroquine (10 μM), or monensin (10 μM). Cells were then incubated with CHX for the indicated times, lysed, and processed for Western blotting.

C, T47D cells were treated for 24 hours with different concentrations of indicated ADCs in the absence or presence of BafA1 (1 μM), and the cell-cycle arrest was evaluated. The experiment was done in triplicate, mean ± StErr.

D, Schematic representation of PRLR, HER2, and PRLR-HER2 chimeric receptors. Full-length PRLR and HER2 are termed PRLR FL and HER2 FL. (Continued on the following page.)
to induce cell-cycle arrest similar in magnitude to that induced by PRLR ADC (Fig. 3F, left). PRLR ADC was not efficient in cells expressing PRLRectoHER2cytoTM (Fig. 3F, left), whereas HER2 ADC caused efficient cell-cycle arrest in cells expressing HER2ctoPRLRcytoTM (Fig. 3F, right).

Taken together, these data demonstrate that the cytoplasmic/transmembrane domain of PRLR is not only responsible for rapid internalization of the receptor (Fig. 3E), but also for efficient ADC-mediated cell-cycle arrest (Fig. 3F).

HER2(T)xPRLR bsAb exploits constitutive lysosomal trafficking of PRLR to promote HER2 degradation

Given the distinct trafficking properties of PRLR and HER2, we hypothesized that bsAbs bridging PRLR and HER2 receptors on the cell surface might promote lysosomal trafficking of HER2 and possibly enhance the activity of HER2 ADC (Fig. 4A). To test this hypothesis, we generated a HER2(T)xPRLR bsAb (HER2(T)xPRLR) containing a HER2 arm from trastuzumab and a PRLR arm from antibody H1H7672P2. HER2(T)xPRLR displayed high-affinity binding to soluble extracellular domains of both HER2 and PRLR (Supplementary Table S1).

As shown in Fig. 4B (left), application of HER2(T)xPRLR induced degradation of HER2, but not of PRLR. PRLR levels did not change because it is constantly being synthesized in endoplasmic reticulum and delivered to plasma membrane (Fig. 4A). HER2 degradation was almost complete 5 hours after HER2(T)xPRLR addition (Fig. 4B, left). Moreover, HER2(T)xPRLR effect on HER2 levels was blocked by Moneosin (Fig. 4B, right), indicating that, similarly to PRLR turnover, HER2(T)xPRLR-induced HER2 degradation is a lysosomal process. In contrast, incubation of T47D cells with PRLR or HER2 antibodies, either separately or combined, did not affect HER2 levels (not shown).

Importantly, no HER2 degradation was detected when binding of either PRLR or HER2 arm of HER2(T)xPRLR was blocked with the respective receptor extracellular domain (Fig. 4C), indicating that degradation of HER2 by HER2(T)xPRLR requires binding of the bsAb to both targets. Finally, we show that HER2(T)xPRLR promotes HER2 degradation in T-REx HEK293 cells expressing PRLR FL (Fig. 4D, top), but not in cells expressing truncated PRLR lacking the entire cytoplasmic domain (Fig. 4D, bottom), indicating that both clustering of the extracellular domains of PRLR and HER2 induced by HER2(T)xPRLR and PRLR turnover mediated by its cytoplasmic domain are required for HER2 degradation.

HER2xPRLR bsAb1 induces lysosomal trafficking of HER2

Next, we wanted to test whether lysosomal trafficking of HER2 ADC could be improved by combining it with HER2xPRLR bsAbs. However, HER2(T)xPRLR could not be used for this purpose because its HER2 arm competes with HER2 ADC for binding to HER2. To circumvent this problem, we generated HER2xPRLR bsAbs with HER2 arms derived from antibodies with epitopes distinct from that of trastuzumab. Eighteen HER2xPRLR bsAbs, each containing one of six HER2 arms, and one of three PRLR arms, were generated as previously described (23). As determined by Western blotting experiments, all 18 HER2xPRLR bsAbs induced degradation of HER2 when added to T47D cells. For instance, as shown in Supplementary Fig. S4, HER2xPRLR bsAbs numbered 1 to 5 mediated HER2 degradation with efficiency similar to that of HER2(T)xPRLR. HER2RxPRLR bsAb1 was selected for further studies.

To test the ability of HER2xPRLR bsAb1 to initiate HER2 lysosomal trafficking, fluorescently labeled PRLR and HER2 antibodies were incubated with T47D cells at 37°C for the indicated times in the presence of either HER2xPRLR bsAb1 (Fig. 5, right) or nonbinding control Ab (Fig. 5, left), whereas the lysosomes were labeled with fluorescent dextrans. Confocal images showed that in the presence of HER2xPRLR bsAb1, but not a nonbinding control Ab, the HER2 antibody signal progressively moved away from the plasma membrane and into dextran-labeled lysosomes (Fig. 5), indicating that HER2xPRLR bsAb1 is capable of inducing lysosomal trafficking of HER2. HER2xPRLR bsAb1 also induced colocalization of HER2 with PRLR (Fig. 5A, right), indicating that HER2, PRLR, and HER2xPRLR bsAb1 traffic to the same lysosomes.

HER2xPRLR bsAb1 enhances HER2 ADC-induced cell-cycle arrest and cell killing, whereas HER2xPRLR bsADC kills HER2+/PRLR- breast cancer cells more potently than HER2 ADC

Because HER2xPRLR bsAb1 was able to enhance HER2 lysosomal trafficking, we wanted to evaluate whether HER2xPRLR bsAb1 could further increase the sensitivity of T47D/HER2 cells to HER2 ADC. To this end, we first examined the effects of different concentrations of HER2 ADC, with or without HER2xPRLR bsAb1, on cell-cycle arrest in T47D/HER2 cells (Fig. 6A). HER2xPRLR bsAb1 augmented the effect of HER2 ADC, with up to 13% cells in early mitosis for the combination treatment (HER2 ADC at 30 nmol/L). For comparison, PRLR ADC induced cell-cycle arrest in T47D/HER2 cells with up to 13% cells in early mitosis at 30 nmol/L ADC (Fig. 6A).

To determine if HER2xPRLR bsAb1 could augment HER2 ADC effect on cell killing, we examined the effects of different concentrations of HER2 ADC on viability of T47D/HER2 cells with or without HER2xPRLR bsAb1. As shown in Fig. 6B, T47D/HER2 cells were moderately sensitive to HER2 ADC, but HER2xPRLR bsAb1 increased the potency of HER2 ADC by reducing the percentage of viable cells at lower ADC concentrations (HER2 ADC + HER2xPRLR bsAb1). As expected, PRLR ADC was highly effective in killing T47D/HER2 cells (0.55 nmol/L IC50).

To validate that HER2xPRLR bsAb1-mediated enhancement of cell killing by HER2 ADC was mediated by DM1 toxin, we
examined DNA integrated intensity of individual nuclei of the same cells used to generate the data in Fig 6B. Supplementary Fig. S5 shows that HER2xPRLR bsAb1 increased HER2 ADC–induced cell-cycle arrest in G2–M phase at 10 nM, 5 nM, and 0.5 nM of ADC. Similar to HER2xPRLR bsAb1, other HER2xPRLR bsAbs enhanced HER2 ADC–mediated cell-cycle arrest and cell killing in T47D/HER2 cells (not shown). In summary, our data indicate that HER2xPRLR bsAbs can induce lysosomal trafficking of HER2 in T47D/HER2 cells and potentiate HER2 ADC–induced cell-cycle arrest and cell killing in T47D/HER2 cells (Fig. 6C).

As an alternative to enhancing the effect of HER2 ADC by combining it with naked HER2xPRLR bsAb, we generated a HER2xPRLR bsADC by conjugating HER2xPRLR bsAb1 to DM1. To determine if bridging HER2 to PRLR with HER2xPRLR bsADC could result in enhanced ADC-mediated cell killing, we compared the effects of different concentrations of HER2 ADC, HER2xPRLR bsADC, and PRLR ADC on the viability of T47D/HER2 breast cancer cells. As expected, HER2xPRLR bsADC (0.4 nM IC₅₀) was dramatically more potent than HER2 ADC judging by reduced percentage of viable cells at lower ADC concentrations, and more potent than PRLR ADC (1 nM IC₅₀) or HER2 ADC + PRLR ADC (0.8 nM IC₅₀) combination (Fig. 6D).

To determine if HER2xPRLR bsADC will be more effective than HER2 ADC in breast cancer models expressing endogenous levels of PRLR and HER2 (Supplementary Table S2), we compared the effects of different concentrations of HER2 ADC, HER2xPRLR bsADC, and PRLR ADC on the viability of BT-483 cells, which express 6.90 × 10⁴ HER2 surface receptors per cell (intermediate-to-low HER2 levels) and approximately 8.0 × 10³ PRLR surface receptors per cell. In accordance with these surface levels, HER2 ADC and PRLR ADC had relatively modest effects on BT-483 cell viability (1.5 nM and 2.5 nM IC₅₀, respectively; Fig. 6E). In contrast, the killing

Figure 4.

PRLR turnover is the driving force directing HER2 to lysosomal degradation when both receptors are bridged using HER2(T)xPRLR bsAbs. A, Schematic representation of a bsAb, HER2(T)xPRLR, bridging HER2 with constitutively internalizing PRLR. B, T47D cells were pretreated for 1.5 hours with DMSO or monensin (10 μM), incubated with HER2(T)xPRLR (10 μg/mL) for the indicated times, lysed, and processed for Western blotting. C, HER2(T)xPRLR (10 μg/mL) or HER2(T)xPRLR (10 μg/mL) preincubated with the soluble extracellular domains of human PRLR or HER2 tagged with MycMycHis (PRLR:mmh or HER2:mmh) at 37°C for 1 hour were added to T47D cells for the indicated times. Cells were then lysed and processed for Western blotting. D, T-REx HEK293 cells induced to express either PRLR FL (top) or truncated PRLR lacking the entire cytoplasmic domain (bottom) were incubated with CHX combined with either HER2 antibody, HER2(T)xPRLR, PRLR antibody, or nonbinding control antibody for the indicated times, lysed, and processed for Western blotting. Note that PRLR lacking the entire cytoplasmic domain runs in multiple bands on SDS-PAGE (bottom).
Figure 5.
HER2×PRLR bsAb1 induced lysosomal trafficking of HER2. T47D cells were labeled with fluorescein-3 kDa dextrans (green) to detect lysosomes and treated with CF594-labeled HER2 (red) and AF405-labeled PRLR (blue) antibodies combined with either nonbinding control antibody, 10 μg/mL (left), or HER2×PRLR bsAb1, 10 μg/mL (right), for the indicated times. For each time point, two representative confocal sections (perinuclear and supranuclear) and the XZ view are shown. Scale, 10 μm. Graph. Colocalization of HER2 with lysosomes was quantified as the percentage of 3 kDa dextrans colocalized with receptor antibody detected at a particular time point (% lysosomes colocalizing with internalized receptor, mean ± SIErr.)
Figure 6.
HER2xPRLR bsAb1 enhances HER2 ADC-induced cell-cycle arrest and cell killing, whereas HER2xPRLR bsADC is more effective than HER2 ADC or PRLR ADC. All experiments were done in triplicates, mean ± S.E.M. A, T47D/HER2 cells were treated for 24 hours with different concentrations of indicated ADCs, or HER2 ADC combined with 10 μg/mL HER2xPRLR bsAb1, and the percentage of early mitotic cells was determined. B, T47D/HER2 cells were treated with different concentrations of indicated ADCs, or ADCs combined with 10 μg/mL HER2xPRLR bsAb1 for 3 days, processed for cell viability assay, and IC50 values were calculated. C, Schematic representation of a bsAb (HER2xPRLR bsAb1) enhancing HER2 ADC-mediated cell-cycle arrest and cell killing by bridging HER2 with constitutively internalizing PRLR. HER2 ADC binds to HER2 at the trastuzumab binding site, but does not internalize efficiently. PRLR drags HER2xPRLR bsAb1, HER2, and HER2 ADC to lysosomes allowing for efficient cell killing. D, T47D/HER2 cells were treated with different concentrations of indicated ADCs, or combination of HER2 ADC + PRLR ADC, processed for cell viability assay, and IC50 values were calculated. E, BT-483 cells were treated with different concentrations of indicated ADCs, or combination of HER2 ADC + PRLR ADC for 14 days, processed for cell viability assay, and IC50 values were calculated. Surface expression levels of PRLR and HER2 in BT-483 cells were determined by quantitative flow cytometry. F, BT-483 cells were treated for 48 hours with different concentrations of indicated ADCs, or combination of HER2 ADC + PRLR ADC, and the percentage of early mitotic cells was determined.
effect was dramatically increased when cells were treated with HER2xPRLR bsADC by reducing percentage of viable cells at lower ADC concentrations. HER2 ADC + PRLR ADC combination (0.4 nM IC50) also increased cell killing in comparison with HER2 ADC or PRLR ADC alone but to lesser extent than HER2xPRLR bsADC (0.15 nM IC50, Fig. 6E), indicating that enhanced efficacy of HER2xPRLR bsADC in BT-483 cells may come in part from monovalent and in part from bivalent interaction of HER2xPRLR bsADC with HER2 and PRLR. This conclusion was further supported by data in Fig. 6F, demonstrating that HER2xPRLR bsADC was more efficient at inducing cell-cycle arrest in BT-483 cells compared with HER2 ADC, PRLR ADC, or HER2 ADC + PRLR ADC combination.

HER2xPRLR bsADC was also significantly more effective than HER2 ADC in another HER2+/PRLR+ breast cancer cell line, MDA-MB-361, which expresses approximately 1.5 × 10^6 HER2 and approximately 8.0 × 10^3 PRLR surface receptors per cell (Supplementary Table S2). Taken together, these data demonstrate that HER2xPRLR bsADC has considerably more potent killing activity than HER2 ADC in PRLR+/HER2+ breast cancer cells, likely due to increased lysosomal trafficking of HER2xPRLR bsAb compared with HER2 antibody.

**Discussion**

Although monoclonal antibodies conjugated with tubulin polymerization inhibitors, ado-trastuzumab emtansine (T-DM1) and brentuximab vedotin, have been approved by the FDA, much remains to be learned about how to select the best targets for ADCs (2, 3, 42). In breast cancers expressing high levels of HER2, experience with T-DM1 has shown that tumor-selective expression of an ADC target and its ability to deliver DM1 to lysosomes are of paramount importance (43). However, despite its ability to effectively kill breast cancer cells expressing high levels of HER2, T-DM1 does not efficiently kill cells expressing low and intermediate levels of HER2, possibly because a relatively small amount of cell surface HER2 reaches lysosomes (14, 15). Recently, we have developed an ADC to PRLR and found that, contrary to HER2, PRLR ADC is efficacious in vitro and in vivo at relatively low surface expression levels of PRLR (M.P. Kelly; unpublished data). In this study, we demonstrated that rapid constitutive turnover of PRLR is the mechanism behind PRLR ADC efficacy. Moreover, we have shown that by bridging constitutively internalizing PRLR with HER2 using bsAbs, HER2 lysosomal degradation can be triggered and HER2 ADC efficacy can be significantly improved.

Rapid constitutive turnover of PRLR may allow for rapid accumulation of PRLR ADC molecules in lysosomes, followed by degradation of the antibody and release of high amounts of lysine-MCC-DM1 leading to cell death. Consistent with our observations, de Goeij and colleagues have recently demonstrated that high turnover of cytokine receptor type 2, Tissue Factor, enabled efficient intracellular delivery of ADC (44). Tissue Factor ADC effectively killed a tumor cell lines expressing as low as 2 × 10^4 target molecules per cell (45). Taken together, our work and published data on Tissue Factor (44, 45) indicate that high turnover proteins selectively expressed in tumors, but not in normal vital cells, may represent attractive ADC targets.

We have shown that HER2(Tx)PRLR bsAbs induced rapid lysosomal degradation of HER2 and that process required both arms of the bsAb. Moreover, additional HER2xPRLR bsAb with HER2 and PRLR arms recognizing various epitopes on HER2 or PRLR were equally effective in degrading HER2, indicating that bsAb-mediated HER2 degradation is not dependent on particular PRLR or HER2 arms, but rather on the ability of the bsAb to bridge the targets.

Though antibody-mediated cross-linking can trigger HER2 internalization and degradation by a mechanism which is not fully understood (46), in our study, we clearly demonstrated that when HER2 and PRLR were bridged using HER2(T)xPRLR, PRLR constitutive turnover mediated by its cytoplasmic domain was the driving force for HER2 lysosomal degradation. Not surprisingly, when a HER2xPRLR bsAb with a non-Trastuzumab arm (HER2xPRLR bsAb1) was used, constitutive turnover of PRLR allowed for lysosomal trafficking of HER2 and significantly enhanced HER2 ADC-induced cell-cycle arrest and cell killing in T47D/HER2 cells.

Recently, de Goeij and colleagues demonstrated that ADC payload delivery can be improved using HER2xCD63 bsAbs; CD63 is a protein that is described to shuttle between the plasma membrane and intracellular compartments (47). Taken together, these data and our findings provide a proof-of-concept for the use of high turnover surface proteins, such as PRLR, or shuttles like CD63, in combination with bsAbs, to enhance ADC efficacy.

Though a combination of HER2-ADC plus HER2xPRLR bsAb may be deployed successfully in some PRLR+/HER2 (IHC2+) breast tumors, an alternative strategy of dual targeting with DM1-conjugated bsAbs may work equally well or even have advantages (48). Indeed, the HER2xPRLR bsADC was more effective than HER2 ADC or PRLR ADC at killing breast cancer cells that coexpress intermediate HER2 (IHC2+) and low PRLR levels, suggesting the possibility that this approach could provide benefit in this subpopulation of breast cancer patients.

In summary, our study opens up the possibility of targeting constitutively internalizing, high turnover proteins (even if expressed at low surface levels) with ADCs and/or of exploiting high turnover proteins, in combination with bsAbs, to enhance the efficacy of ADCs targeting proteins that do not efficiently traffic to lysosomes.

**Disclosure of Potential Conflicts of Interest**

M.P. Kelly is Staff Scientist at Regeneron Pharmaceuticals. T. Nittoli has ownership interest (including patents) in Regeneron Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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References

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Bispecific Antibodies and Antibody–Drug Conjugates (ADCs) Bridging HER2 and Prolactin Receptor Improve Efficacy of HER2 ADCs

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