Caveolae-Mediated Endocytosis as a Novel Mechanism of Resistance to Trastuzumab Emtansine (T-DM1)

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Abstract

Trastuzumab emtansine (T-DM1) is an antibody–drug conjugate (ADC) that has demonstrated clinical benefit for patients with HER2+ metastatic breast cancer; however, its clinical activity is limited by inherent or acquired drug resistance. The molecular mechanisms that drive clinical resistance to T-DM1, especially in HER2+ tumors, are not well understood. We used HER2+ cell lines to develop models of T-DM1 resistance using a cyclical dosing schema in which cells received T-DM1 in an “on-off” routine until a T-DM1–resistant population was generated. T-DM1–resistant N87 cells (N87-TM) were cross-resistant to a panel of trastuzumab-ADCs (T-ADCs) with non–cleavable-linked auristatins. N87-TM cells do not have a decrease in HER2 protein levels or an increase in drug transporter protein (e.g., MDR1) expression compared with parental N87 cells. Intriguingly, T-ADCs using auristatin payloads attached via an enzymatically cleavable linker overcome T-DM1 resistance in N87-TM cells. Importantly, N87-TM cells implanted into athymic mice formed T-DM1 refractory tumors that remain sensitive to T-ADCs with cleavable-linked auristatin payloads. Comparative proteomic profiling suggested enrichment in proteins that mediate caveolae formation and endocytosis in the N87-TM cells. Indeed, N87-TM cells internalize T-ADCs into intracellular caveolin-1 (CAV1)–positive puncta and alter their trafficking to the lysosome compared with N87 cells. T-DM1 colocalization into intracellular CAV1-positive puncta correlated with reduced response to T-DM1 in a panel of HER2+ cell lines. Together, these data suggest that caveolae-mediated endocytosis of T-DM1 may serve as a novel predictive biomarker for patient response to T-DM1. Mol Cancer Ther; 17(1); 1–11. ©2017 AACR.

Introduction

Antibody–drug conjugates (ADCs) are emerging therapeutic modalities strategically designed to selectively deliver chemotherapeutic agents to tumors while limiting non-tumor tissue toxicities. ADCs are biotherapeutics compromised of a tumor-targeting antibody and a cytotoxic payload attached via a chemical linker. Once bound to their target antigen, ADCs are internalized and release their cytotoxic payload. Since the early 2000s, three ADCs have received FDA-approval and over 50 more are currently in clinical development (1, 2).

Despite encouraging signs of early clinical benefit, the sustained efficacy of many targeted anticancer therapies is limited by either inherent or acquired tumoral drug resistance. The molecular mechanisms that drive clinical drug resistance are multifactorial and ultimately depend upon the therapeutic modality and cancer indication (3). Modifications of the drug target (e.g., kinase mutations; refs. 4, 5), drug efflux protein expression (e.g., ABCB1 in AML; refs. 6–8), and shifts in the expression ratio of pro-versus antiapoptotic proteins (e.g., BCL-2 expression in AML; ref. 9) are examples of tumor alterations that correlate with reduced clinical response to anticancer therapies. However, the molecular mechanisms that drive clinical resistance to ADCs remain largely unknown.

Trastuzumab emtansine (T-DM1, Kadcyla®) currently represents the only FDA-approved ADC for the treatment of a solid tumor indication. T-DM1 is composed of the trastuzumab antibody chemically bound to the microtubule-disrupting DM1 maytansinoid via a thioether linker (10). In the phase III EMILIA clinical trial, T-DM1 showed significantly improved progression-free and overall survival versus lapatinib plus capecitabine in HER2+ metastatic breast cancer patients who had failed previous treatment with trastuzumab and a taxane (11). The overall response rate for HER2+ patients treated with T-DM1 was 43.6% (11). On the basis of these results, T-DM1 was granted FDA approval for the treatment of this patient population. However, these data showed that, despite high tumoral HER2 positivity, over 50% of patients treated with T-DM1 did not show clinical benefit. In the phase II/III GATSBY clinical trial in HER2+ gastric cancer patients, T-DM1 did not show enhanced clinical response versus standard chemotherapy (12).

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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expression does not solely predict clinical response to T-DM1 and the need exists to more accurately define the patient sub-population most likely to respond to T-DM1.

Although data are still emerging from T-DM1–treated patient samples, multiple pre-clinical models of T-DM1 resistance have been generated to characterize the changes at the tumor cell level that may mediate clinical resistance to T-DM1. Although the models of T-DM1 resistance in these studies were generated with different dosing schemas, the following are mechanisms responsible for T-DM1 resistance seen across multiple models: (i) decreased HER2 expression (13, 14), (ii) increased expression and activity of drug efflux proteins (13, 14), and (iii) increased heregulin expression (15).

Herein, we used an ADC structurally similar to T-DM1 (13) to generate T-DM1–resistant cells in vitro. We report the generation and characterization of three models of T-DM1 resistance, including one with a unique trafficking defect that has not been previously associated with T-DM1 acquired resistance and may provide a novel biomarker for clinical response to T-DM1.

Materials and Methods

Cell lines

NCI-N87 gastric carcinoma, HCC1954 breast carcinoma, and BT474 breast carcinoma cells were obtained from the ATCC and maintained in RPMI media supplemented with 10% FBS, 1% l-glutamine, and 1% sodium pyruvate. H69 and H69AR cell lines were obtained from the ATCC and were maintained in RPMI/25 mmol/L HEPES media supplemented with 10% FBS, 1% l-glutamine, and 1% sodium pyruvate. KB, KB8.5, and KBVI cells were generously provided by Dr. Michael Gottesman (National Cancer Institute). Parental N87 cells were purchased from the ATCC (CRL-5822, lot 5803347) in June 2010. N87 and N87-TM cell lines were authenticated as NCI-N87 in 2014 (IDEXX BioResearch), when the cells were being characterized for this work. Mycoplasma testing was conducted approximately quarterly using MycoAlert (Lonza), and results were consistently negative; the most recent testing was conducted in October 2016.

Bioconjugations

ADCs and chemical structures of disclosed payloads described herein were prepared as previously described (13). The syntheses and structures of the novel auristatin linker-payloads as well as preparation and characterization of ADCs presented herein are described in the Supplementary Methods.

Generation of T-DM1–resistant cells

Cells were passaged into two separate flasks and each flask was treated identically with respect to the resistance generation protocol to enable biological duplicates. Cells were exposed to five cycles of T-DM1 conjugate at 10-fold IC_{50} concentrations (10 nmol/L payload concentration; ~388 ng/mL antibody concentration) for 3 days, followed by approximately 4 to 11 days recovery without treatment. After five cycles at 10 nmol/L of the T-DM1 conjugate, the cells were exposed to six additional cycles of 100 nmol/L T-DM1 in a similar fashion. The procedure was intended to simulate the chronic, multi-cycle (on/off) dosing at maximally tolerated doses typically used for cytotoxic therapies in the clinic, followed by a recovery period. Parental cells derived from NCI-N87 are referred to as N87, and cells chronically exposed to T-DM1 are referred to as N87-TM. Moderate- to high-level drug resistance developed within 4 months for N87-TM cells. Drug selection pressure was removed after approximately 3 to 4 months of cycle treatments when the level of resistance no longer increased after continued drug exposure. Responses and phenotypes remained stable in the cultured cell lines for approximately 3 to 6 months. Thereafter, a reduction in the magnitude of the resistance phenotype as measured by cytotoxicity assays was occasionally observed, in which case early passage cryopreserved T-DM1–resistant cells were thawed for additional studies. All reported characterizations were conducted after removal of T-DM1 selection pressure for at least 2 to 8 weeks to ensure stabilization of the cells. Data were collected from various thawed cryopreserved populations derived from a single selection, over approximately 1 to 2 years after model development to ensure consistency in the results. Clonal populations were derived from single N87-TM cells using a limited dilution technique. Briefly, single cells were plated into each well of a 96-well plate and cultured in the presence of 100 nmol/L T-DM1 until clonal populations were established.

Cytotoxicity assay

Cellular responses to ADCs and free drugs were measured with a cytotoxicity assay as previously described (13). Briefly, cells were seeded into 96-well plates and treated with varying concentrations of ADCs or free drugs for 96 hours. Cell viability was measured using CellTiter 96 AQueous One MTS Solution (Promega). IC_{50} values were calculated using a four-parameter logistic model with XLfit (IDBS).

Proteomic profiling and immunoblot analyses

Proteomic profiling of membrane fractions was performed and analyzed as previously described (13). Whole-cell lysates were prepared in RIPA buffer supplemented with protease inhibitor cocktail (Sigma). Lysates were fractionated on 4% to 20% SDS-PAGE gels (Bio-Rad), transferred onto membranes and probed with primary and secondary antibodies further detailed in the Supplementary Methods.

Immunofluorescence and live-cell imaging

For immunofluorescence experiments, cells were seeded onto #1.5 coverslips (Electron Microscopy Services) and fixed with 4% paraformaldehyde. Cells were stained with primary antibodies at room temperature (RT) for 1 hour and secondary antibodies at RT for 30 minutes. Coverslips were mounted onto slides with Pro-Long Diamond Antifade Mountant (Thermo Fisher Scientific). Images were acquired with a Zeiss LSM 710 (Carl Zeiss Inc.). For imaging cytometry, cells were incubated with PE-labeled trastuzumab (2 µg/mL) on ice for 90 minutes or at 37°C for time points indicated. At designated time points, internalization was stopped by addition of cold PBS and cells were fixed with Cytofix/Cytoperm solution (BD Biosciences) for 20 minutes. Cells were immunostained with LAMP1-AF647 (BD Biosciences; cat# 562622) for 30 minutes or CAV1 antibody for 30 minutes followed by an anti-rabbit IgG Fab2 AF647 secondary antibody (ThermoFisher Scientific; cat# A-21246) for 30 minutes. Cells were analyzed on the ImageStreamX Mark II Imaging Flow Cytometer (AMNIS, EMD Millipore). For live-cell imaging experiments, cells were seeded into 4-well CELView dishes (Grenier Bio-One) and imaged with an ImageEM camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan) mounted onto a CSU-X1 spinning disk head (Yokogawa Electric Corporation, Inc.) on an
Eclipse Ti microscope stand (Nikon Instruments Inc.) equipped with a 60x oil objective (NA1.4, Nikon Instruments Inc.). Image analysis was performed using Velocity 3D Image software (PerkinElmer).

**In vivo efficacy studies**

All procedures performed on animals in this study were in accordance with established guidelines and regulations, and were reviewed and approved by the Pfizer Institutional Animal Care and Use Committee. Pfizer animal care facilities that supported this work are fully accredited by AAALAC International. Female NOD scid gamma (NSG) immunodeficient mice (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) were obtained from The Jackson Laboratory. Mice were injected subcutaneously in the right flank with suspensions of either N87 or N87-TM cells (7.5 × 10^6 cells per injection, with 50% Matrigel). Mice were randomized into study groups when tumors reached approximately 0.3 g (~250 mm^3). ADCs or vehicle were administered intravenously in saline on day 0 and repeated for a total of four doses, 4 days apart (q4d × 4). Tumors were measured with calipers weekly and tumor mass calculated as volume = (width × width × length)/2.

**Immunohistochemistry**

Five micrometer–thick formalin-fixed, paraffin-embedded tissue sections were stained immunohistochemically. Detailed sample preparations and staining procedures are provided in the Supplementary Methods.

**Results**

**Generation and characterization of T-DM1–resistant cells**

To identify potential mechanisms that may drive clinical resistance to T-DM1 ADC, we generated T-DM1–resistant versions of the HER2-expressing cancer cell lines N87, BT474, and HCC1954. We used a cyclical dosing schema of a 3-day exposure to high dose T-DM1 ADC followed by a washout and recovery period to mimic patient exposure and pharmacokinetics when they receive bolus chemotherapy treatment cycles (Fig. 1A).

N87 cells were inherently sensitive to T-DM1 (IC50 = 1.6 nmol/L payload concentration; 62 ng/ml antibody concentration; Fig. 1B). Two populations of N87 cells were exposed to the treatment cycles and, after approximately four months of cyclical dosing, these two populations (henceforth named N87-TM-1 and N87-TM-2) became refractory to the ADC by 109-fold compared with parental N87 cells (Fig. 1B, Table 1). Interestingly, minimal cross-resistance (1.8x) to the corresponding unconjugated maytansinoid free drug, DM1-Sme, was observed (Supplementary Fig. S1, Table 1) which suggests the alterations in N87-TM cells that lead to T-DM1 resistance are specific to the whole ADC biomolecule and not solely to the payload.

Previous reports of T-DM1 resistance in other cell models have implicated ATP-binding cassette (ABC) transporter protein overexpression (e.g., ABCB1/MDR1, ABCG2/MRP1) or target antigen downregulation as key drivers of T-DM1 resistance (13, 14). However, proteomics and immunoblots demonstrate that N87-TM cells do not overexpress MDR1 (Fig. 1C) or MRP1 (Fig. 1D) and retain similar levels of HER2 as compared with parental N87 cells (Fig. 1E). Despite retaining high levels of HER2, N87-TM cells display approximately 50% reduced binding to the unconjugated antibody of T-DM1 (i.e., trastuzumab) as measured by flow cytometry (Supplementary Fig. S2). In contrast, T-DM1–resistant populations of BT474 (BT474-TM) and HCC1954 (HCC1954-TM), selected in a similar manner as N87-TM, show significantly decreased HER2 expression (Supplementary Fig. S3A and S3B), suggesting that reduced antigen is the primary mode of resistance in these two cell models. Hence, we focused additional characterization on the N87-TM model.

Taking advantage of the modular nature of ADCs, we hypothesized that altering key components of the biomolecule would result in an agent able to overcome T-DM1 resistance in N87-TM cells. N87-TM cells displayed cross-resistance to several trastuzumab-based ADCs (T-ADCs; Fig. 1F and Table 1) which were conjugated to auristatins via a non-cleavable maleimide linker. Remarkably, N87-TM cells were sensitive to T-ADCs conjugated to auristatins using a protease-cleavable linker sequence (ValCitr-PAB) in an antigen-dependent manner (Fig. 1G, Supplementary Fig. S4 and Table 1, Supplementary Table S1). The sensitivity of N87-TM cells to other trastuzumab-based ADCs suggests that reduced levels of HER2 in these cells is not a primary mode of resistance.

To determine whether the ADC-resistant N87-TM cells were broadly resistant to other standard-of-care chemotherapeutics, N87 and N87-TM cells were treated with DNA-damaging agents (e.g., cisplatin, doxorubicin), kinase inhibitors (e.g., rapamycin, dacomitinib), and microtubule inhibitors (e.g., docetaxel, vinblastine). Importantly, N87-TM cells remained sensitive to these small molecule inhibitors when compared with the parental N87 cells, discounting general growth defects as a mechanism of resistance to T-DM1 ADC (Supplementary Fig. S4, Table 1).

**N87-TM tumors maintain T-DM1 resistance in vivo**

A problem that often plagues in vitro drug resistance models is their lack of translatability into the in vivo setting. To determine whether the resistance observed in cell culture was recapitulated in vivo, N87 cells and N87-TM cells were injected into the flanks of female NOD scid gamma (NSG) immunodeficient mice and treated with T-DM1 ADC [at doses of 6 mg/kg (mpk) and 10 mpk)] and a T-ADC with a cleavable-linked auristatin (T-vc-Aur0101; at 3 mpk), on a q4d × 4 schedule. The N87 tumors retained high expression of HER2 in vivo and were quite sensitive to all three T-ADC treatment conditions (Fig. 2A). Remarkably, N87-TM tumors also retained high expression of HER2 in vivo and were refractory to both regimens of T-DM1 ADC, yet tumors regressed with T-vc-Aur0101 (Fig. 2B). Thus, N87-TM tumors retain a similar response profile to T-DM1 and T-vc-Aur0101 ADCs in vitro and in vivo.

To better understand how N87-TM tumors are resistant to T-DM1 and sensitive to T-vc-Aur0101, we first interrogated the tumoral distribution of the ADCs. N87-TM tumors, from the previously described efficacy study, were collected 24 hours following the last ADC dose, and ADC distribution was evaluated through immunohistochemistry staining with an anti-human IgG antibody. N87-TM tumors treated with 6 mpk T-DM1 ADC displayed almost complete tumor coverage of IgG binding, whereas those treated with 3 mpk T-vc-Aur0101 only had ADC binding at the most vessel-proximal regions of the tumor (Supplementary Fig. S5). Thus, tumoral ADC distribution is unlikely to explain the efficacy response of these ADCs in N87-TM tumors.

Next, we evaluated a biomarker of downstream response to an anti-microtubule–targeted agent to determine how T-DM1 and T-vc-Aur0101 ADCs differentially impacted N87 and N87-TM tumors. Phosphorylation of histone H3 (pHH3) is a marker
Figure 1.
N87-TM model generation and characterization. A, Schematic model of cyclical dosing paradigm used to generate T-DM1 resistant cells. Parental cells were dosed with T-DM1 for 3 days, followed by a washout and recovery period. This process was cycled multiple times until a T-DM1 resistant population emerged. B, Parental N87 (closed circle) and two T-DM1-resistant populations (open square and open circle), selected simultaneously (N87-TM-1 and N87-TM-2), were treated with T-DM1 for 4 days. Cell viability is plotted against ADC payload concentration. C–E, Whole-cell lysates from parental and T-DM1-resistant N87 cells were separated by SDS-PAGE and levels of MDR1 (C), MRP1 (D), and HER2 (E) were determined by immunoblot analyses. Whole-cell lysates from positive and negative control cell lines for MDR1 (KB85 and KBV1) and MRP1 (A549 and H69AR) are shown. F, Parental and T-DM1-resistant N87 cells were treated with a trastuzumab-ADC conjugated to a non-cleavable linker and auristatin payload for 4 days. Cell viability is plotted against ADC payload concentration. G, Parental and T-DM1-resistant N87 cells were treated with a trastuzumab-ADC conjugated to a cleavable-linked auristatin payload for 4 days. Cell viability is plotted against ADC payload concentration.
of mitotic cells and can be used to evaluate the activity of antimitotic agents. To determine whether N87-TM tumors had reduced pHH3 staining following T-DM1 ADC treatment as compared with N87 tumors, mice were administered a single-dose of T-DM1 ADC (6 mpk) and tumors were collected 24 hours later. Co-immunostaining revealed ADC and pHH3 double-positive cells in the N87 tumors (Fig. 2C, top left), whereas many of the ADC-bound N87-TM tumor cells were pHH3 negative (Fig. 2C, bottom left). These data are consistent with the efficacy data shown when N87, but not N87-TM, tumors are sensitive to T-DM1 ADC. In a similar study conducted with T-vc-Aur0101 ADC showing N87, but not N87-TM, tumors are sensitive to a T-ADC with a cleavable linker but with a released species of cysteine-mc-Aur8261 (Supplementary Fig. S6). Thus, T-ADCs whose active catabolite is an amino acid-capped linker payload are effective in killing N87, but not N87-TM, cells. Taken together, these data show that N87-TM tumors maintain T-DM1 resistance in vivo and T-ADCs with cleavable linkers and permeable payloads overcome T-DM1 resistance in vitro and in vivo.

The role of CAV1 in ADC biology and resistance
To further characterize the mechanism of T-DM1 resistance in N87-TM cells in an unbiased approach, we performed a proteomic evaluation of membrane fractions to profile proteins differentially expressed in the N87-TM cells as compared with the parental N87 cells. Significant changes in expression level of 523 proteins between the cell lines were observed (Fig. 3A). Immunoblot analysis validated a number of the proteomic hits (Fig. 3B). Of interest was the overexpression of caveolin-1 (CAV1) at Tyr 14 (21). Indeed, N87-TM cells have enhanced phospho-Src and phospho-CAV1 as compared with N87 cells (Fig. 3C). Immunofluorescence for CAV1 in N87 and N87-TM cells is shown in Figure 3D. Immunofluorescence for CAV1 in N87 and N87-TM cells is shown in Figure 3D.

Table 1. Resistance profiles of N87 and N87-TM cells to ADCs, standard-of-care chemotherapeutics and other unconjugated drugs

<table>
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<tr>
<th>Compound</th>
<th>Linker Type</th>
<th>N87 (IC50, nmol/L)</th>
<th>N87-TM (IC50, nmol/L)</th>
<th>Relative Resistance</th>
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<tr>
<td>T-MCC_DM1</td>
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<td>1.6</td>
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<tr>
<td>T mc_Aur8261</td>
<td>NC</td>
<td>5.3</td>
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<td>&gt;188x</td>
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<tr>
<td>T mc_MMAD</td>
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<td>16</td>
<td>492</td>
<td>31x</td>
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<tr>
<td>T mc_Aur0101</td>
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<td>138</td>
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<td>&gt;7x</td>
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<tr>
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NOTE: IC50 values of ADCs reported here represent payload concentrations. Data are mean of multiple determinations. These relative resistance values are represented graphically in Supplementary Fig. S4. Relative resistance is the ratio of the mean IC50 value for the TM-resistant cell line versus the parental cell line. Abbreviations: C, cleavable; NC, noncleavable.
cells demonstrates that N87-TM cells contain many more intracellular CAV1⁺ puncta than N87 cells (Fig. 3D).

We next hypothesized that N87-TM cells can internalize T-DM1 into these caveolar endocytic compartments. We treated N87 and N87-TM cells with T-DM1 for 24 hours and co-immunostained for anti-human IgG and CAV1 to interrogate the co-localization of the ADC with CAV1⁺ puncta. Indeed, T-DM1 ADC co-localized with CAV1 in N87-TM, but not N87, cells (Fig. 3E). To determine whether this positive correlation between T-DM1 ADC and CAV1 occurred at the early phases of ADC endocytosis, we analyzed trastuzumab and CAV1 co-localization at multiple early time points using imaging cytometry. N87-TM, but not N87, cells showed enhanced trastuzumab and CAV1 co-localization over time (Fig. 3F).

Because the internalization of trastuzumab was into differential compartments in N87 and N87-TM cells, we next sought to determine whether N87-TM cells displayed impaired lysosomal trafficking of trastuzumab because T-DM1 is catabolized in the lysosome. Lysosomal co-localization of trastuzumab was decreased in N87-TM as compared with N87 cells (Fig. 3F).

Caveolar endocytic compartments are phenotypically distinct from the endo-lysosomal pathway (22). For example, the lumina of caveolar endocytic compartments have a neutral pH, whereas the lumina of endo-lysosomal compartments are increasingly acidic during pathway maturation (23). To determine whether T-ADCs were internalized into compartments with different pH, we generated a T-ADC conjugated to two fluorescent moieties: (i) a pH-insensitive AlexaFluor-modified auristatin non-cleavable linker payload and (ii) a pH-sensitive dye, pHRodo, whereby the fluorescence signal is proportional to the acidity of the micro-environment. We performed live-cell imaging with this conjugate.
Figure 3.

N87-TM cells are enriched for intracellular caveolar compartments and internalize trastuzumab-ADCs via caveolae-mediated endocytosis. 

A, Membrane fractions from N87 and N87-TM cells were prepared for proteomic profiling. A volcano plot is shown representing the relative fold change for each protein (x-axis) by the significance level for each protein. Points in the upper right quadrant of the plot represent proteins enriched in N87-TM cells as compared with N87 cells.

B, Whole-cell lysates of N87 and N87-TM cells were prepared, separated by SDS-PAGE and immunoblotted for proteins identified in the proteomic profiling to validate the hits.

C, Whole-cell lysates from N87 and N87-TM cells were separated by SDS-PAGE and levels of total SRC, phospho-SRC, total CAV1, and phospho-CAV1 were determined by immunoblot analysis.

D, CAV1 puncta were identified via immunofluorescence staining of N87 and N87-TM cells. Average of number of puncta calculated as total number of CAV1 puncta normalized by number of nuclei for each field of view.

E, N87 and N87-TM cells were treated with T-DM1 overnight at 37°C, fixed and co-immunostained with anti-human IgG (green color) and anti-CAV1 (red color). Image analysis was performed to determine co-localization between T-DM1 and CAV1 in N87 and N87-TM cells.

F, N87 and N87-TM cells were incubated with fluorescently labeled trastuzumab and kinetic co-localization analysis between T-DM1 and CAV1 and LAMP1 markers were performed via image cytometry.
flourescent T-ADC monitoring ADC internalization into pHrodo-positive areas within the cell. As expected, T-ADC showed increasingly higher co-localization with low pH compartments through the time course in N87 cells (Supplementary Fig. S7A, black line). However, the T-ADC did not traffic into low pH compartments in N87-TM cells during the same time course (Supplementary Fig. S7A, red lines). Using the pH-insensitive AlexaFluor signal intensity as a normalization factor, the distribution of T-ADC into low pH compartments was decreased in N87-TM cells as compared with N87 cells (Supplementary Fig. S7B).

Caveolae-mediated ADC internalization is likely to be the predominant mechanism by which T-ADCs enter N87-TM, but not N87, cells. To determine whether we could induce caveolae-mediated ADC internalization in N87 cells, we transfected CAV1-GFP into N87 cells and performed live-cell imaging. CAV1-GFP introduction resulted in the formation of similar intracellular CAV1+ puncta as N87-TM cells (Supplementary Fig. S8). Interestingly, T-ADC containing an AlexaFluor-modified auristatin non-cleavable linker payload internalized into the induced caveolar endocytic compartments in N87 cells (Supplementary Fig. S8).

These data suggest that T-ADC internalization in N87-TM cells is mediated via caveolar endocytosis. We next hypothesized that this caveolar-mediated endocytosis drives resistance to T-DM1 in N87-TM cells. To determine whether CAV1 knockdown re-sensitized N87-TM cells to T-DM1, we performed doxycycline (DOX)-controlled shRNA-mediated CAV1 knockdown in N87-TM cells. Following DOX withdrawal, CAV1 targeted shRNAs were expressed and resulted in reduced CAV1 protein levels as compared with a non-targeted control sequence (Supplementary Fig. S9). However, CAV1 knockdown was not sufficient to re-sensitize N87-TM cells to T-DM1 (Supplementary Fig. S9). Taken together, these data suggest that N87-TM cells are enriched for caveolar endocytic compartments capable of internalizing T-DM1 and differentially providing an alternative route for ADC endocytosis and trafficking.

Caveolae-mediated T-DM1 endocytosis across HER2+ cell lines

Caveolae-mediated endocytosis has been implicated as a possible mechanism of inherent insensitivity to ADCs targeting other antigens (e.g., melanotransferrin; ref. 24). We investigated a panel of HER2+ cell lines to determine their inherent sensitivity to T-DM1 and whether CAV1 protein level correlated with the magnitude of T-DM1 response. We did not observe a positive correlation between CAV1 protein level and decreased T-DM1 sensitivity in this panel of HER2+ cell lines (Fig. 4A). Given that high levels of CAV1 protein alone do not necessarily induce the formation of caveolar endocytic compartments (25), we next determined the propensity for caveolar-mediated T-DM1 internalization across this cell line panel. Interestingly, some CAV1 high cell lines (e.g., JIMT1, N87-TM) showed colocalization of T-DM1 and CAV1+ puncta whereas other CAV1 high cell lines (e.g., SKOV3, HCC1954) did not (Fig. 4B; similar data observed with T-vc-Aur0101, Supplementary Fig. S10). Intriguingly, the amount of ADC colocalization with CAV1+ puncta positively correlated with reduced response to T-DM1 across this cell line panel (Fig. 4C). Collectively, these data suggest that the inherent proclivity of tumor cells to internalize T-DM1 via caveolar-mediated endocytosis may predict their response to the ADC.

Discussion

In this study, we generated and characterized multiple in vitro models of T-DM1 resistance using a drug selection paradigm designed to mimic the clinical dosing regimen of T-DM1. Using this dosing schema, two models (BT474-TM and HCC1954-TM) acquired T-DM1 resistance via a previously reported mechanism (i.e., decreased HER2 expression) and another model (N87-TM) acquired T-DM1 resistance likely via a novel mechanism of differential ADC internalization and trafficking. Herein, we report the first model of acquired T-DM1 resistance that implicates caveolar-mediated endocytosis of T-DM1 as a novel mechanism of T-DM1 resistance.

Despite using the same approach to generate resistance, these three models and our two previously reported models of T-DM1 resistance (13) highlight the diversity of molecular mechanisms that cells may adopt to overcome the challenge of chronic drug exposure. Although the background genetics of each model may dictate the eventual mechanism of resistance to a drug, one might expect a myriad of changes that may impact a tumor’s sensitivity to T-DM1 given the complexity of its mechanism of action. For T-DM1 to effectively kill a tumor cell, it must (i) bind to HER2; (ii) internalize into the intracellular portion of the cell; (iii) transit through the endosomal maturation pathway with final delivery to the lysosome; (iv) be catabolized within the lysosomal milieu releasing a membrane impermeable payload that is likely transported out of the lysosome via a lysosomal membrane transporter to interact with tubulin (the target of DM1). Indeed, there are examples of potential alterations to some of these steps in experimental models that may ultimately lead to T-DM1 resistance. As previously mentioned, decreased HER2 expression could prevent sufficient T-DM1 binding but other factors have been shown to influence trastuzumab binding to HER2, including the accumulation of the truncated p95-HER2 isoform that lacks a trastuzumab binding site (26). Efficient internalization of T-DM1 may not always be achieved because trastuzumab-HER2 complexes have been shown to have a rapid rate of plasma membrane recycling once they are internalized (27). It remains to be seen whether a similar phenomenon occurs with T-DM1-HER2 complexes. Lysosomal delivery of T-DM1 is another key event and the results of this study show that alternative T-DM1 internalization and trafficking parameters can influence lysosomal accumulation of T-DM1. The mechanism of lysosomal escape of lysine-MCC-DM1 (the released species of T-DM1) has long been debated but the recent description of SLC46A3 as a lysosomal membrane transporter protein that mediates the lysosome-to-cytoplasm transfer of this metabolite has provided insights into potential mechanisms of transport (28).

Despite the complexity of these biomolecules, the modular nature of ADCs allows for the interfering of various components to assess their influence on the properties of the ADC. The T-DM1–resistant N87-TM cells were cross-resistant to trastuzumab–ADCs delivering other non-cleavable linked tubulin inhibitors. By switching the linker to an enzymatically cleavable linker, ADCs carrying other microtubule-targeting payloads (e.g., ValCita-linked auristatins) were able to overcome T-DM1 resistance in N87-TM cells. In a similar fashion, cell line models made resistant to CD22- or CD79b-targeting ADCs employing the vcMMAE linker-payload remained sensitive to the respective ADCs delivering a different payload (e.g., PNU-159682; ref. 29). Thus, by swapping each module of an ADC with a different functional
group, ADC activity can be modulated. This “component switching” property of ADCs implies that patients with ADC-refractory tumors may still derive benefit from a similar ADC, targeting the same antigen, but with altered composition.

This report implicates caveolae-mediated endocytosis of T-DM1 as a potential mechanism of acquired resistance, yet trastuzumab-ADCs delivering tubulin inhibitors with an enzymatically cleavable linker overcame T-DM1 resistance. It is important to note that caveolae contain enzymatically-active cysteine cathepsins capable of processing the “ValCit” linker utilized herein (30). Thus, although the caveolar microenvironment may not favor the antibody catabolism necessary to release the non-

Figure 4.
Caveolae-mediated endocytosis of T-DM1 predicts reduced sensitivity to T-DM1. A, A panel of breast and gastric cancer cell lines were evaluated for their relative HER2 and CAV1 at basal state by immunoblot analysis. CAV1 score was assigned on the basis of basal CAV1 expression from this experiment. The same panel of cell lines was tested for sensitivity to T-DM1 when treated for 4 days in a cell viability assay. The correlation of CAV1 score versus the T-DM1 IC_{50} value is represented. B, The panel of cell lines were incubated with T-DM1 and analyzed for co-localization to CAV1 puncta as previously described in Fig. 3. Representative images from each cell line show T-DM1 (green color) and CAV1 (red color) immunostaining. White arrows indicate examples of T-DM1 co-localized with CAV1 puncta (yellow/orange color). C, The T-DM1 and CAV1 co-localization scores from (B) for each cell line were plotted against the IC_{50} value of each cell line to T-DM1. The correlation between T-DM1 and CAV1 colocalization score versus the T-DM1 IC_{50} value is represented.
cleavable linker-payload of T-DM1, it may be suitable to process enzymatically cleavable linkers. The impact of caveolaemediated endocytosis of other ADCs has been reported as well. Melanotransferrin- and CD133-targeting ADCs showed potent cytotoxic activity in antigen-positive cell lines where the ADC preferentially accumulated in lysosomes; however, the conjugates were not active in antigen-positive cell lines in which the ADC colocalized with caveolin-1 (24, 31). Both of these ADCs used the "vcMMAF" linker-payload which contains the same enzymatically cleavable dipeptide linker used here but releases a different payload (monomethyl auristatin E, MMAE). Because of its carboxylic acid functional group, MMAF is a charged species at neutral pH; thus, although the linker may be processed in caveola, the charged nature of MMAF renders it membrane impermeable and not able to enter into the cytoplasm of the cell. It is this property that prevents ADCs using MMAF from having substantial bystander effect (2). Along similar lines, a CD20-targeting ADC using a "vcMMAE" linker was reported to be internalized via both clathrin- and caveolaemediated endocytosis; however, the nature of endocytosis did not affect the sensitivity of antigen-positive cell lines to the ADC (32). Unlike MMAF, monomethyl auristatin E (MMAE) does not exist as a charged molecule at neutral pH and is membrane permeable. Data herein implicate caveolaemediated endocytosis in T-DM1 resistance; however, another group has suggested that caveolaemediated endocytosis may sensitize cells to T-DM1 (33). Although these data implicate a possible role for CAV1, the inherent differences in sensitivity to free DM1 (18) may explain the differences in basal sensitivity to T-DM1 seen across the high HER2 cell lines evaluated in that study.

The functional studies in this report implicate a role for caveolaemediated endocytosis in the mechanism of resistance to T-DM1 in N87-TM cells. However, CAV1 knockdown was not sufficient to re-sensitize N87-TM cells to T-DM1. Incomplete CAV1 protein loss may prevent re-sensitization, or as eluded to previously, the formation of caveola is not strictly dictated by the level of CAV1 expression, but rather the interplay of caveolaemembrane coat proteins (caveolin family) interacting with caveolaemembrane stabilizing scaffold proteins (cavin family; ref. 20). N87-TM cells are enriched for proteins from both families (i.e., CAV1 and PTRF). Thus, it may be that the driver of caveola formation in N87-TM cells is a combination of altered proteins. Indeed, another caveola coat protein may be compensating for the loss of CAV1 in the CAV1-knockdown N87-TM cells which, in conjunction with the enhanced expression of PTRF, allows for the continued formation of caveola. Along similar lines, CAV1 expression solely did not correlate with T-DM1 sensitivity across a panel of HER2 + cell lines; rather, the functional aptitude for cells to internalize/traffic T-DM1 into caveolar compartments correlated with T-DM1 sensitivity. The function of caveola in cancer biology remains largely unknown. However, this report adds to the growing body of literature that suggests caveola may dictate sensitivity of cancer cells to ADCs that use certain types of linker-payloads.

It has become increasingly clear that "precision medicine" will only be as effective as our ability to define patient sub-populations through the use of reliable biomarkers. For many targeted therapies, such as ADCs, the paramount biomarker is the expression of the target for that agent (e.g., HER2 for T-DM1). However, given tumoral genetic complexity and heterogeneity, target expression does not solely predict a patient's tumor sensitivity to a targeted therapy, particularly in solid tumor indications. For example, in the EMILLA trial, only 43% of breast cancer patients with high HER2-expressing tumors responded to T-DM1 (11). Thus, there continues to be an unmet need in our ability to better identify patients most likely to respond to T-DM1 and other targeted therapies. Although alterations in some genes (e.g., EGFR, HER3, and PTEN protein expression and PIK3CA mutations; ref. 34) have been found to not correlate with T-DM1 response, data in this report warrant the investigation of caveolaemediated endocytosis of T-DM1 in patient tumors as a potential predictive biomarker for response to T-DM1 and other non-cleavable-linked ADCs.

**Disclosure of Potential Conflicts of Interest**
Russell Dushin has ownership interest (including patents) in Pfizer Stock and Stock Options. No other potential conflicts of interest were disclosed.

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**References**

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