***Loganzo, et al, Tumor cells chronically treated with a trastuzumab-maytansinoid antibody-drug conjugate develop varied resistance mechanisms but respond to alternate treatments***

**Supplementary Information:**

1. **Supplemenatry Materials & Methods**
2. **Supplementary Tables**
3. **Supplementary Figure Legends**
4. **Supplementary Figures (See separate PDF file)**

**1. Supplementary Materials & Methods:**

**ADCs and compounds:** Antibody-drug conjugates were prepared by Pfizer as indicated in the bioconjugation method section below. Unconjugated maytansine analog (DM1-SMe) and auristatin analog (Aur-8254) were prepared by Pfizer Worldwide Medicinal Chemistry (Groton, CT). Structures of key compounds are represented in Supplementary Fig. S2. Aur-8261 (the free-acid form of Aur-8254) is reported as compound 22a in Maderna et al. (24). Other standard-of-care chemotherapeutics were purchased from Sigma (St. Louis, MO).

**Bioconjugations:** Trastuzumab-maytansinoid conjugate (TM-ADC) is structurally similar to trastuzumab emtansine (T-DM1, or Kadcyla®). TM-ADC is comprised of anti-Her2 trastuzumab antibody covalently bound to DM1 through the bifunctional linker sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC). Sulfo-SMCC is first conjugated to the free amines on the antibody for one hour at 25ºC in 50 mM potassium phosphate, 2 mM EDTA, pH 6.8, at a 10:1 reaction stoichiometry, and unbound linker is then desalted from the conjugated antibody. This antibody-MCC intermediate is then conjugated to DM1 sulfide at the free maleimido end on the MCC linker antibody overnight at 25ºC in 50 mM potassium phosphate, 50 mM NaCl, 2 mM EDTA, pH 6.8, at a 10:1 reaction stoichiometry. Remaining unreacted maleimide is then capped with L-cysteine, and the ADC is fractionated through a Superdex200 column to remove non-monomeric species (21). The ADC was further characterized via SEC for purity, LC-MS to calculate drug-antibody loading (DAR), and the concentration was determined via UV spectrophotometry. The DAR of TM-ADC conjugates used for cell selections and treatments was in the range of 3.5 – 4.2. The TM-ADC used in these studies is intended to represent trastuzumab antibody with non-cleavable-linked mertansine (DM1) as a representative antibody-drug conjugate structurally similar to Kadcyla®, which was not yet approved or available upon initiation of these studies. Analytical, in vitro, and in vivo comparisons of TM-ADC and Kadcyla®indicate that these agents are mechanistically identical, hence this approach allowed the generation of resistant models prior to the availability of Kadcyla®. Auristatin containing ADCs were prepared by partial reduction of the antibody followed by reaction of reduced cystine residues with desired maleimide terminated linker-payload. Specifically, antibodies were partially reduced via addition of 2.2 – 3.0 molar excess of tris(2-carboxyethyl)phosphine (TCEP) in 100 M HEPES buffer, pH 7.0, and 1 mM diethylenetriaminepentaacetic acid (DTPA) for 2 h at 37°C. The desired linker-payload was then added to the reaction mixture at a linker-payload/mAb-thiol molar ratio of 7 and reacted for an additional 1 h at 25 °C in the presence of 15% (v/v) of dimethylacetamide (DMA). After the 1 h incubation period, *N*-ethylmaleimide (NEM) was added to cap the unreacted thiols and allowed to react for 15 min followed by addition of 6 fold excess L-Cys to quench any unreacted linker-payload. For T\_mc’\_Aur-8261 and T\_Mal’Peg6C2\_MMAD, hydrolysis of the succinimide ring was conducted via incubating the purified ADC in a 100 mM borate, pH 9.2, buffer for 24-72 hr at 37°C, as recently reported (25). Hydrolyzed versions of these linkers are indicated as mc’ and Mal’ to distinguish from non-hydrolyzed versions mc and Mal, as these forms had higher potency in parental JIMT1 cells and were used as representative examples of non-cleavable-linked ADCs. For all conjugations, the reaction mixtures were dialyzed overnight at 4°C in PBS, pH 7.4, and purified via size-exclusion chromatography (SEC) on an AKTA Explorer (General Electric) with Superdex 200 10/30 GL column. ADCs were further characterized by size exclusion chromatography (SEC) for purity, and by hydrophobic interaction chromatograph (HIC) and liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI MS) to calculate drug loading, and protein concentration was determined via UV spectrophotometry. Structures of ADCs are represented in Supplementary Fig. S2B (non-cleavable-linked) and S2C (cleavable-linked), where “mAb” denotes the trastuzumab antibody.

**Procedure to make cell lines resistant to TM-conjugate (additional information)**: Cells were exposed to multiple cycles of TM conjugate at approximately IC80 concentrations for 3 days, followed by approximately 4 to 11 days recovery without treatment. The procedure was intended to simulate the chronic, multi-cycle (on/off) dosing at maximally tolerated doses typically used for cytotoxic therapeutics in the clinic, followed by a recovery period (Supplementary Fig. S1). Parental cells derived from MDA-MB-361-DYT2 are referred to as 361, and cells chronically exposed to TM-ADC are referred to as 361-TM. JIMT1 cells chronically exposed to TM-ADC are referred to as JIMT1-TM. In some cases, the JIMT1 parental cell model was inherently less sensitive to several ADCs, hence it was not possible to assess the effect of these agents in the resistant JIMT1-TM population. Moderate- to high-level drug resistance developed within 1.5 to 3 months for 361-TM and JIMT1-TM cells. Drug selection pressure was removed after ~3 - 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 – 6 months thereafter. We occasionally observed a reduction in the magnitude of the resistance phenotype as measured by cytotoxicity assays, in which case early passage cryo-preserved TM-ADC resistant cells were thawed for additional studies. All reported characterizations were conducted after removal of TM-ADC selection pressure for at least 2 - 8 weeks to ensure stabilization of the cells. Data were collected from various thawed cryopreserved populations derived from a single selection, over approximately 1 – 2 years after model development to ensure consistency in the results.

**Induction of Her2 expression:** Induced expression of Her2 was conducted in JIMT1-TM cells by infection with a retroviral construct for human Her2 generously provided by Dr. Timothy Fisher, Pfizer Oncology (La Jolla, CA). Briefly, HEK293T cells were co-transfected with retrovirus packaging vector (pCL-10A1) and pPLPCX-huHer2 in OptiMEM for 6 hr, then maintained in DMEM for 48 hr. Virus-containing media was collected, 0.45 µm filtered, and frozen until use. Viral infection of JIMT1-TM cells was conducted with 1 mL of virus stock, 8 µg/mL polybrene, and MEM with 10% serum for 24 hr. Cells were selected with 2 µg/mL puromycin for several weeks, and whole cell lysates analyzed for Her2 expression by immunoblot.

**Knockdown of ABCC1 via siRNA transfection:** ON-TARGET plus SMARTpool human ABCC1 (MRP1) siRNA and non-specific control siRNA were obtained from GE Dharmacon (Lafayette, CO). One day prior to transfection, 361-TM resistant cells were seeded into 6-well plates at 2.5 x 105 cells/well so that cells reached 50 - 70% confluence by the next day. Transfection was performed according to the manufacturer’s instructions. Briefly, siRNA and Lipofectamine RNAiMAX reagent (Life Technologies, Grand Island, New York) were diluted into Opti-MEM medium, followed by combining diluted siRNA and diluted Lipofectamine RNAiMAX reagent at 1:1 ratio, incubating at room temperature for 15 min to form complexes, then 250 ul siRNA-lipid complexes were added directly into cultured cells in 2 ml media per well. The final concentration of siRNA was 20 nM and the amount of Lipofectamine RNAiMAX reagent was 7.5 ul per well. After 48 hr transfection, cells were analyzed by re-plating into 96-well plates at 3000 cells /well, the next day treated with ADC. After 96 hr, cells were harvested as described in the “Cytotoxicity Assay” method section.

**Live cell imaging microscopy:** Cells (2.5 x 105) were seeded into 35 mm dishes with glass bottoms (MatTek Corporation, Ashland, MA; cat# P35G-1.0-14-C). The next day, lysosomes were-labeled by adding Alexa-Fluor-647-Dextran, 10,000 MW (Life Technologies, Carlsbad, CA; cat# 22914) at final concentration 125 µg/mL and cultured overnight. On the third day, cells were incubated with 10 µg/mL BODIPY -labeled ADC in PBS on ice for 45 min. The labeled ADCs included Cys-conjugated anti-Her2-trastuzumab-mcValCitPABC-Aur-BODIPY (cleavable linker) and anti-Her2-trastuzumab-mc-Aur-BODIPY (non-cleavable linker). After incubation, cells were washed 3X with cold PBS to remove unbound ADC, then replenished with cold growth media. The imaging dish was immediately moved to a Zeiss LSM710 confocal microscopy workstation for live cell imaging at 37°C/5% CO2. Image acquisition was at 10 min intervals for 15 hr to monitor ADC trafficking over time. Volocity imaging software (PerkinElmer, Waltham, MA) was used to analyze co-localization of internalized ADC (BODIPY signal) with lysosomal marker (Alexa-Fluor-647 signal). Data were expressed in terms of Pearson’s Correlation Coefficient (PCC). Statistical significance between parental and resistant cell lines was evaluated by comparing mean PCCs between parental and resistant lines at 10 minute intervals from 0 to 960 minutes post-dose using repeated-measures analysis of variance (ANOVA) methods. Cell type (parental/resistant) and time were main effects in the ANOVA model, which included a cell type-by-time interaction term and a compound symmetry covariance structure to account for within-sample correlation over time. Separate variance and covariance estimates were calculated for parental and resistant cell types. The dependent variable for the ANOVA was the inverse of (PCC+1) to better conform with distributional assumptions underlying ANOVA. Pairwise comparisons between cell types at each time point were based on t-tests using the ANOVA error estimate. P-values were not adjusted for multiple comparisons, but were only interpreted as indicative of substantive differences if they remained low across multiple time points.

**Receptor recycling studies:**  Receptor recycling was measured utilizing an antibody feeding technique as previously described (30). Briefly, JIMT1 and JIMT1-TM cells were incubated with an anti-transferrin receptor antibody or TM-ADC (10 µg/mL) for 30 minutes at 4°C and then incubated for 30 minutes at 37°C to allow internalization of antibody-bound receptor complexes. Residual surface-bound antibody was acid stripped (0.5 M NaCl and 0.2 M acetic acid) for 2 min at 4°C. Any remaining surface-bound antibody was blocked with a 10-fold molar excess of unconjugated secondary antibody for 30 minutes at 4°C. The cells were then incubated with an AlexaFluor-488 conjugated secondary antibody for 60 min at 4°C and 37°C to visualize receptors that had recycled back to the plasma membrane. The cells were then fixed (4% paraformaldehyde and 4% sucrose), permeabilized (0.1% Triton X-100) and stained with an AlexaFluor-555 conjugated secondary antibody to visualize internalized receptors that had not recycled back to the plasma membrane. The extent of recycling is measured as the recycling index which is determined by a ratio of the average fluorescence of recycled receptors to internalized receptors. Images were acquired with a Zeiss LSM 710 using a 63X 1.4NA Plan-Apochromat objective.

**Released species metabolomic studies:** The released species from cell-processed ADCs were identified by methanol extraction of cell pellets followed by LC-MRM analysis. Briefly, 0.5 mL of 80% MeOH was added to cell pellets and the resulting suspension incubated at room temperature for 15 min. Aliquots (10 µl) of the first suspension solution were taken for BCA protein assay and protein amounts were used to normalize the differences in cell numbers among treatments. The pooled supernatant was centrifuged at 15,000 x g at 4°C for 12 min and a 30 µl aliquot was injected for LC-MRM analysis. LC-MRM analysis was performed on an Agilent 1200 HPLC coupled with AB SCIEX API4000 mass spectrometer. The released species were separated with an Imtakt Scherzo SM-C18 150 × 2.0 mm column, 3 µm particle size with 13 nmol/L pore size, with mobile phase A of 100% water containing 0.1% formic acid, and mobile phase B of 100% acetonitrile containing 0.1% formic acid with the flow rate of 0.3 mL/min at column oven of 30°C. The following gradient was used: 20% B for 3 min, 20%-95% B for 20 min, 95% B for 2 min. The released species were ionized with heated ESI probe in the positive ionization mode with the following source parameters: TEM=550, CUR=20, GS1=60, GS2=60, IS=5500, CAD=5, and detected with multiple reaction monitor (MRM) mode. EP was set as 10 and DP, CE and CXP were tuned for each species. MRM transitions for possible released species MMAD, vcMMAD, Cys\_vc-MMAD, Mal’Peg6C2\_MMAD, Cys\_MalPeg6C2\_MMAD and Cys\_Mal’Peg6C2\_MMAD are listed in Supplementary Figure S10E and F. Ring-open hydrolyzed (water adduct) maleimide groups are denoted as Mal’. The detected species by LC-MRM were further confirmed by spiking-in the respective purified standards.

**2. Supplementary Tables**

**Supplementary Table S1: Properties of cell lines made resistant to TM-conjugate.**

|  |  |  |
| --- | --- | --- |
|  | TM-ADC | Maytansinoid free drug (DM1-SMe) |
| Cell line | IC50, nmol/L (IC50, µg/mL Ab) | Relative Resistance | IC50, nmol/L | Relative Resistance |
| 361 (parental) | 1.6 nmol/L± 1.3 (26)(0.06 µg/mL) | **-** | 11 nmol/L± 5.1 (6) | **-** |
| 361-TM | 410 nmol/L± 110 (23)(14 µg/mL) | **256 X** | 42 nmol/L± 13 (6) | **3.8 X** |
| JIMT1 (parental) | 52 nmol/L± 33 (20)(1.8 µg/mL) | **-** | 16 nmol/L± 4.8 (6) | **-** |
| JIMT1-TM | 820 nmol/L± 310 (18)(27 µg/mL) | **16 X** | 55 nmol/L± 29 (5) | **3.4 X** |

Data represent cytotoxicity analyses after cells had undergone >5 cycles of chronic TM-ADC therapy over the course of approximately 1 year. IC50 values are the mean ± standard deviation (n, number of determinations), and represent nmol/L payload and µg/mL of antibody (in parentheses) for conjugate, and represent nmol/L payload for free drug. Relative Resistance is the ratio of the mean IC50 for the TM-ADC resistant cell line vs. the corresponding parental cell line.

**Supplementary Table S2:**

**Relative resistance of selected free payloads and Cys-capped-released species in H69 (no ABCC1) and H69AR (high ABCC1 expressing) cell lines**

|  |  |  |  |
| --- | --- | --- | --- |
| Compound Name | H69 (mean IC50, nmol/L) | H69AR (mean IC50, nmol/L) | Relative Resistance (RR) |
|
|
| doxorubicin | 76 | 1700 | **22.4** |
| DM1-SMe | 1.9 | 78 | **41.1** |
| MMAD | 0.12 | 0.41 | **3.4** |
| Cys\_mc\_MMAD | 570 | 3900 | **6.8** |
| Cys\_MalPeg6C2\_MMAD  | 360 | 2500 | **6.8** |
| Cys\_mc\_Aur-8261 | 3200 | >10000 | **> 3.1** |
| Cys\_MalPeg6C2\_Aur-8261 | 4300 | >10000 | **> 2.3** |
| TM-ADC | 210 | 2000 | **9.5** |

Data are mean IC50 of 2 – 6 independent experiments, rounded to 2 significant figures. Relative resistance is the ratio of IC50 for the H69AR vs. H69 cell lines. These cells do not express Her2 antigen, hence the IC50 for TM-ADC is higher than in Her2-positive cells, yet some resistance is still observed in these high MRP1-expressing cells.

**Supplementary Table S3: Patterns of resistance, conjugate trafficking, and released products in TM-ADC resistant cell lines.**

|  |  |  |  |
| --- | --- | --- | --- |
|  |  | Non-cleavable LP | Cleavable LP |
| Cell Model | Her2 protein changes | Resistance Profile | Increased lysosomal co-localization? | Reduced released species? | Resistance Profile | Increased lysosomal co-localization? | Reduced released species? |
| **361-TM** | **↓** | **+** | **+** | **+** | **-** | **-** | **+** |
| **JIMT1-TM** | **↓↓** | **+** | **+** | **++** | **+** | **+** | **++** |

361-TM and JIMT1-TM cell lines were incubated with ADCs containing representative non-cleavable or cleavable linker-payloads (LP) and cells evaluated by cytotoxicity, live image trafficking, or metabolomic assessment of released products from ADC processing. Each observation is scored qualitatively for levels of Her2 protein (↓, ~25% decrease;↓↓, ~58% decrease),cytotoxicity resistance profile (-, sensitive; +, resistant), increased co-localization of BODIPY-labeled ADC with lysosome (-, low or + high), and reduction of released species compared to parental cells (-, no, +, low; ++, moderate).

**3. Supplementary Figure Legends**

**Supplementary Figure S1: Schematic illustration of the approach used to create trastuzumab-maytansinoid ADC resistant cell lines.** Breast cancer cell lines were treated with TM-ADC at doses where ~80% cell death is typically observed in short term cytotoxicity assays. MDA-MB-361-DYT2 cells were treated with 125 nmol/L TM-ADC by cycling (3 days exposure, followed by recovery of approximately 4 to 11 days) over several months. JIMT1 cells were treated with 500 nmol/L TM-ADC using a similar approach. Populations of cells which survived chronic exposure were named 361-TM and JIMT1-TM, respectively, and were further characterized.

**Supplementary Figure S2: Structures of payloads and ADCs.** Structures of payloads (A), ADCs with non-cleavable linkers (B), and ADCs with cleavable linkers (C) which are described in the text. DM1-SMe is a surrogate permeable payload for TM-ADC in cytotoxicity assays since the released species, Lys-MCC-DM1, is impermeable. Similarly, auristatin Aur-8254 (methyl-ester) is the permeable version of Aur-8261 (carboxylic acid), which differ only in their C-terminus. Note that in our ADC nomenclature, the “underscore” denotes the separator between antibody\_linker\_payload components, and that “mAb” indicates the antibody component of the ADC; the remaining structure is linker-payload. Each ADC has approximately 4 linker-payloads per antibody.

**Supplementary Figure S3: 361-TM-resistant cells show differential responses to ADCs with modified linkers, payloads, and antibodies.** 361-parental cells (*open circles*) and 361-TM resistant cells (*closed squares*) **(**A to D), or JIMT1-parental (*open circles*) and JIMT1-TM resistant cells (*closed squares*) (E to H),were treated with the indicated ADCs. (A, E) trastuzumab ADC containing a non-cleavable linker and auristatin (T\_mc’\_Aur-8261); (B, F) trastuzumab ADC containing a non-cleavable linker and auristatin (T\_Mal’Peg6C2\_Aur-8261); (C, G) trastuzumab ADC containing cleavable linker plus MMAD (T\_vc\_MMAD); (D, H) trastuzumab ADC containing cleavable linker plus auristatin (T\_vc\_Aur-8254). Cytotoxicity was assessed after 4 days treatment. Data are representative of multiple experiments. Error bars are within the size of the symbols for some data points. Average IC50 (nmol/L of payload) from multiple experiments for the indicated cell line and ADC is indicated in each graph.

**Supplementary Figure S4: Ectoptic expression of Her2 in JIMT1-TM cells rescues sensitivity to TM-ADC.** JIMT1-TM cells were infected with retroviral construct coding for human Her2 protein. (A) Immunoblot demonstrates that Her2 was induced in JIMT1-TM+Her2 cells, to levels greater than in parental JIMT1 cells. JIMT1 (*closed square*), JIMT1-TM (*open circle*), and JIMT1-TM+Her2 (*closed triangle*) cell lines were treated for 4 days with the indicated ADCs, TM-ADC (B), and T\_mc’\_Aur-8261 (C), and analyzed for survival by MTS endpoint.

**Supplementary Figure S5: Cell surface proteomic profiling of 361-TM resistant cells, with evidence of increased ABCC1.** Volcano plot depiction of proteins altered in 361 vs. 361-TM cell lines. Arrow indicates ABCC1 (MRP1). Y-axis, p-value; x-axis, log2-fold change. Data were updated so that 0 values were replaced by 0.5, normalized by column sum, and log2-transformed. The resulting normalized log2-transformed spectral count data were analyzed using Statistical Analysis of Microarrays (SAM) implemented in R. The top scoring proteins when filtering on a p-value ≤ 0.05 are shown as red; non-significant scoring proteins are shown as grey.

**Supplementary Figure S6: Knockdown of ABCC1 (MRP1) in 361-TM cells sensitizes to ADCs.** 361-TM cells were transfected with siRNA to knock-down ABCC1 mRNA. ABCC1 protein levels are indicated in the immunoblot (A). Cells were treated with TM-ADC (B) and T\_Mal’Peg6C2\_MMAD (C). ADC IC50 (nmol/L payload) in the presence of non-targeting control siRNA or ABCC1 siRNA are indicated in the figure.

**Supplementary Figure S7: Endosomal and lysosomal proteins altered in JIMT1-TM resistant cells as determined by proteomic profiling.**

(A) Pairwise comparison of JIMT1 and JIMT1-TM proteins limited to those in the endosome (GO:005768) and lysosome (GO:0005764) gene sets (Gene Ontology in the Cancer Genome Anatomy GO Browser). Plot are generated from spectral count data, where spectral counts are updated so that 0 values are replaced by 0.5, normalized by column sum, analyzed using Statistical Analysis of Microarrays (SAM) implemented in R. Fold-change represents log2-transformed values for JIMT1-TM vs. JIMT1. The top scoring proteins are shown when filtering on a p-value of 0.5. Y-axis, –log10 p-value; x-axis, log2-fold change. (B) List of all lysosome and endosome proteins from the JIMT1-TM vs. JIMT1 comparison with fold-change greater than 2. “Primary ID” indicates database identifier. “Gene id” is entrez gene identifier. “Gene symbol” is the official gene symbol. “Description” is the gene description taken from WebGestalt. “Fold-change” is magnitude of protein increase as measured in JIMT1-TM vs. JIMT1 data set. “P-value” is the p-value calculated as described in the SAM analysis. “Category” is the GO-associated gene set for each protein.

**Supplementary Figure S8: Immunoblot verification of elevated surface-associated proteins in JIMT1-TM resistant cells**. Both whole cell lysates (*left panel*) and biotin-enriched surface membrane proteins (*right panel*) from JIMT1-TM and JIMT1 parental cells were probed for proteins originally detected by proteomics profiling, including RABGAP1, PAK4, and HECTD1. For some proteins, increases were observed in the membrane associated fraction but not in the whole cell lysate.

**Supplementary Figure S9: Co-localization analyses of ADC and lysosome via live-cell imaging.** 361 and JIMT1 parental and TM-ADC-resistant cell lines were incubated with fluorescently labeled ADCs containing either non-cleavable (MC) or cleavable (VC) linker and conjugate trafficking was monitored by live cell microscopy. Data are mean Pearson coefficient, which is indicative of the magnitude of co-localization of the respective labeled ADC linker-payload with the lysosomal compartment. Data at each time point is the mean of n= 1 to 3 independent studies. \* Denotes time points where Pearsons coefficient of resistant cells was significantly different by ANOVA (P<0.05) from parental cells for a span of at least 3 minutes of continuous measurements.

 (A) 361 parental cells treated with cleavable-linked T\_mcValCitPABC\_Aur-BODIPY (VC linker) or with non-cleavable-linked T\_mc\_Aur-BODIPY (MC linker);

(B) 361 and 361-TM cells incubated with ADC containing VC linker;

(C) 361 and 361-TM cells incubated with ADC containing MC linker;

(D) JIMT1 parental cells treated with T\_mcValCitPABC\_Aur-BODIPY (VC linker) or with T\_mc\_Aur-BODIPY (MC linker);

(E) JIMT1 and JIMT1-TM cells incubated with ADC containing VC linker;

(F) JIMT1 and JIMT1-TM cells incubated with ADC containing MC linker. n=1 run for JIMT1 and n=2 runs for JIMT1-TM so no significance reported;

(G) Representative images of 361, 361-TM, JIMT1, and JIMT1-TM cells at 0 hr and approximately 6 hr after T\_mc\_Aur-BODIPY ADC addition to the cells. Arrows in “6 hr Merge” column indicate co-localization of ADC with lysosome.

**Supplementary Figure S10: Altered trafficking in JIMT1-TM cells are specific to TM-ADC and not transferrin receptor antibody.** (A) JIMT1 and JIMT1-TM cells were incubated live with an anti-transferrin receptor antibody or TM-ADC (at 10 µg/mL) and receptor-bound complexes were assessed as described in the Supplementary Methods section. Representative images of the 60 minute incubation are shown. Scale bars, 20 µm. (B) Quantification of transferrin receptor antibody and TM-ADC recycling in JIMT1 and JIMT1-TM cells. Recycling Index is defined as the fluorescence intensities of recycled receptors divided by internalized receptors following image thresholding. Data represent means ± SEM of the Recycling Index. \*\*p <0.01; Mann-Whitney test.

**Supplementary Figure S11: LC-MRM analyses of released products from ADCs incubated with parental and resistant cell lines.** 361 and JIMT1 parental and TM-ADC-resistant cell lines were incubated with either non-cleavable-linked conjugate T\_Mal’Peg6C2\_MMAD or with cleavable-linked conjugate T\_mcValCitPABC\_MMAD, cells harvested after 36 hr, and released payload products were extracted from cell pellets and subjected to LC-MRM analysis for the respective putative released payload products. (A) Levels of Cys\_Mal’Peg6C2\_MMAD in 361 and 361-TM cells upon incubation with non-cleavable-linked ADC. (B) Levels of MMAD in 361 and 361-TM cells upon incubation with cleavable-linked ADC. (C) Levels of Cys\_Mal’Peg6C2\_MMAD in JIMT1 and JIMT1-TM cells upon incubation with non-cleavable-linked ADC. (D) Levels of MMAD in JIMT1 and JIMT1-TM cells upon incubation with cleavable-linked ADC; quantification of released species was conducted for MMAD since a reference standard was available: 361 cells, 3.0 pmol of MMAD; 361-TM, 2.5 pmol; JIMT1, 1.1 pmol; JIMT1-TM, 0.7 pmol.

(E) Multiple reactive monitoring (MRM) transitions (Q1/Q3, precursor/fragment) for the respective putative released payload products from non-cleavable ADCs.

(F) MRM transitions (Q1/Q3, precursor/fragment) for the respective putative released payload products from cleavable ADCs.

**4. Supplementary Figures**

See separate PDF file.