Aberrant intracellular metabolism of T-DM1 confers T-DM1 resistance in human epidermal growth factor receptor 2-positive gastric cancer cells

Hongbin Wang,1,2 Wenqian Wang,1 Yongping Xu,1 Yong Yang,1 Xiaoyan Chen,1 Haitian Quan1 and Liguang Lou1

1Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai; 2University of Chinese Academy of Sciences, Beijing, China

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Correspondence
Haitian Quan and Liguang Lou, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 555 Zuchongzhi Road, Shanghai 201203, China. Tel.: +86-21-5080 6056; Fax: +86-21-5080 7088; E-mails: haitianquan@simm.ac.cn; lglou@mail.shcnc.ac.cn

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Gastric cancer is one of the most frequent human malignancies and the second-leading cause of cancer-related deaths worldwide. There is mounting evidence that HER2 (ErbB2) overexpression is important in patients with gastric and gastroesophageal junction cancer.

Trastuzumab, a humanized mAb against HER2, has been approved, in combination with chemotherapy, as a new standard option for patients with HER2-positive advanced gastric or gastroesophageal cancer.

Trastuzumab emtansine (T-DM1), an ADC comprising the HER2-targeted antibody trastuzumab and the antimicrotubule agent mertansine (DM1), a derivative of maytansine, containing a non-cleavable linker was approved by the FDA in February 2013 to treat HER2-positive metastatic breast cancers.

Binding of T-DM1 to HER2 triggers internalization of the HER2–T-DM1 complex into the cell through receptor-mediated endocytosis. The DM1-containing metabolite, lysine-MCC-DM1, which is produced through lysosome-dependent proteolytic degradation of T-DM1, plays a major role in the tumor activity of T-DM1 through inhibition of microtubule assembly, which ultimately causes cell death.

Because the non-cleavable linker is stable in both the circulation and the tumor microenvironment, release of active DM1 occurs only as a result of lysosome degradation in cells, a property ensured by the activity of V-ATPase, which achieves a highly acidic pH (≤5) that promotes optimal activity of various hydrolases and vesicular transport. Despite favorable initial outcomes, most HER2-positive patients treated with T-DM1 remain incurable because of the ultimate development of acquired resistance. In addition, some HER2-positive cancers are primarily non-responsive or are only minimally responsive to T-DM1.

Thus, understanding resistance mechanisms and exploring strategies for overcoming T-DM1 resistance are urgent priorities.

In the present study, we used HER2-positive N87 gastric cancer cells to establish a T-DM1-resistant cell line termed N87-KR. We found that aberrant activity of V-ATPase in lysosomes of N87-KR cells results in defects in T-DM1 metabolism and thus a decrease in the T-DM1 metabolite, leading to
failure to inhibit microtubule polymerization and, ultimately, T-DM1 resistance. Moreover, H-MMAE, another HER2-targeted ADC containing a cleavable linker, was able to overcome T-DM1 resistance in N87-KR cells. Thus, we propose that V-ATPase activity in lysosomes may be a novel biomarker for predicting T-DM1 resistance, and further suggest that ADCs with cleavable linkers may be used to overcome T-DM1 resistance in patients with decreased tumor lysosome V-ATPase activity.

Materials and Methods

Reagents and antibodies. Both T-DM1 and trastuzumab were purchased from F. Hoffmann-La Roche (Basel, Switzerland). T-DM1 was provided by Jiangsu Hengrui Pharmaceutical Co. (Lianyungang, China). Bafilomycin A1 was obtained from Sel- leck Chemicals (Houston, TX, USA). DyLight 488 NHS Ester and LysoTracker Deep Red were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Propidium iodide, sulforhodamine B, and DAPI were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acridine orange was purchased from China National Pharmaceutical Industry Corp (Beijing, China). Hertuzumab-vc-MMAE was obtained from Rongchang Pharmaceuticals, Ltd (Yantai, China). Antibodies against HER2, GAPDH, and PARP were purchased from Cell Signaling Technology (Beverly, MA, USA). The antibody specific for β-tubulin was purchased from Sigma-Aldrich. The antibody against β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alexa Fluor 488-conjugated goat anti-mouse IgG was purchased from Invitrogen (Carlsbad, CA, USA).

Cell culture and treatment. The N87 cell line was obtained from ATCC (Manassas, VA, USA). Cells were cultured according to the instructions provided by ATCC, and were tested and authenticated by Genesky Biotechnologies (Shanghai, China).

N87-KR cells were established by chronic exposure of N87 cells to gradually increasing concentrations of T-DM1 from initial 50 ng/mL to ultimate 1 μg/mL. After 18 months, the resistant cells were selected as polyclonal T-DM1-resistant N87KR cells and T-DM1-resistant monoclonales were selected through the limiting dilution method.

Cell proliferation assay. Cell proliferation was determined by sulforhodamine B assay, as described previously.

Western blot analysis. Western blotting was carried out as described previously. In brief, cells were lysed in SDS sample buffer, then separated by SDS-PAGE, and transferred to PVDF membranes (Millipore, Bedford, MA, USA). Membranes were incubated with primary antibodies at 4°C overnight and then with secondary antibodies for 2 h at room temperature. Immunoreactive proteins were detected using the Western blot image system from Thermo Fisher Scientific.

Cell cycle analysis. Cells were collected and fixed in ice-cold 70% ethanol overnight at −20°C. After staining with propidium iodide, the DNA content of cells was measured using a FACSscan flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed with FlowJo 7.6 software.

Polymeric tubulin fraction assay. After drug treatment, cells were lysed and extracted for 3 min at room temperature with buffer consisting of 80 mM MES-KOH (pH 6.8), 1 mM MgCl₂, 1 mM EGTA, 0.1% Triton X-100, 10% glycerol, and protease inhibitors. The detergent-insoluble fraction containing cytoskeletal polymerized microtubules was analyzed by Western blotting.

Binding, endocytosis, and recycling assays using cell-surface fluorescence quenching. Binding assay. Cells on 6-well plates were surface-labeled by incubating on ice for 1 h with DyLight 488 NHS Ester-linked T-DM1 (1 μg/mL). After washing three times with cold FBS buffer (2% FBS/PBS), cells were incubated on ice for 15 min with trypsin, treated with an equal volume of FBS, sedimented at 1000 g, and analyzed for binding affinity by flow cytometry.

Endocytosis assay. After pre-binding T-DM1 by incubating on ice for 1 h, cells on 6-well plates were incubated in growth medium at 37°C for the indicated intervals to allow internalization of surface fluorescence, after which cells were rapidly chilled, detached, and sedimented as previously described.

Cells were then incubated on ice for 15 min with stripping buffer (ddH₂O, 0.05 M glycine [MW 75.07] pH 2.45, and 0.1 M NaCl) to quench surface fluorescence, and then washed three times with FACS buffer. Internalized fluorescence was analyzed immediately by flow cytometry.

Exocytosis assay. After pre-incubation with T-DM1 on ice for 1 h to allow binding, as described for the endocytosis assay above, cells in 6-well plates were incubated at 37°C for 1 h, then quickly chilled, detached, and incubated with stripping buffer on ice for 15 min. After washing three times with FACS buffer, cells were warmed to 37°C for the indicated intervals, then rapidly chilled, detached, surface-quenched, and analyzed by flow cytometry.

Fluorescence microscopy. Cells were seeded overnight on 6-well plates (5 x 10⁵ cells/well) containing a cover glass. Labeled T-DM1 was added, and the cells were incubated at 37°C for 24 h, with the lysosome fluorescent probe Lyso-Tracker Red (50 nM) added 1 h prior to observation. The culture medium was then removed, and cells were fixed with 4% paraformaldehyde for 15 min. For immunocytochemical detection of microtubule polymerization, cells were first fixed with 4% paraformaldehyde, and then permeabilized with 0.1% Triton X-100 for 5 min. Thereafter, cells were incubated first with anti-tubulin antibody (diluted 1:200 in 2% BSA/PBS) at 37°C for 2 h, and then with Alexa Fluor 488-conjugated goat anti-mouse IgG (diluted 1:200 in 2% BSA/PBS) for 1 h at 37°C. The nuclear compartment was stained with DAPI by incubating on ice for 10 min. All subsequent wash steps were carried out using PBS. Cells were imaged with a 60 × oil-immersion objective using an Olympus FV1000 confocal microscope (Tokyo, Japan).

Lysine-MCC-DM1 metabolite analysis. N87 and N87-16-8 cells were cultured in 6-well plates, treated with 10 μg/mL T-DM1 alone or with 10 μg/mL T-DM1 plus 1 nM Baf-A1, and incubated for the indicated time or 24 h. Cells were collected, washed three times with PBS, and then centrifuged at 1000 g for 10 min. The identities and concentrations of T-DM1 metabolites in precipitated cells were determined by HPLC/MS. Cells were disrupted and extracted by adding acetonitrile, and then ultrasonicated. Cell fragments were removed by centrifugation, and proteins in the supernatant were precipitated by adding 25 μL internal standard (IS) solution (levonorgestrel, 200 ng/mL) and 200 μL methanol to a 50-μL aliquot of the supernatant. The mixture was mixed by vortexing for 1 min and then centrifuged for 1 min at 14 000 g. The upper layer was injected for LC-MS/MS analysis.

The LC-MS/MS detection was carried out using an LC30AD ultra-fast LC system (Shimadzu, Kyoto, Japan) coupled to a Triple Quad 5500 tandem mass spectrometer (Sciex, Foster City, CA).
CA, USA) equipped with a Turbolon Spray source. Chromatographic separation was carried out on a Triart C18 column (50 × 2.1 mm i.d., 1.9 µm; YMC; Shimadzu Corp., kyoto, Japan). The mobile phase used for gradient elution consisted of 5 mM ammonium acetate : aqueous formic acid (100:0.2, v/v) and acetonitrile. Mass spectrometer detection was operated in the positive multiple reactions monitoring mode.

In vivo study. Female nude mice (BALB/cA-nude, 5–6 weeks old) were purchased from Shanghai SLAC Laboratory Animal Co. (Shanghai, China). A tumor model was created by s.c. implanting 5 × 10⁶ N87 or N87-16-8 cells into nude mice. Forty-eight hours after inoculation, mice were randomized into six groups and treated with vehicle (60% PEG-400), T-D1M (10 mg/kg, i.v.), or H-MMAE (3 mg/kg, i.v.) once for a total of 21 days. Tumor volume was calculated as width² × length × 0.5, and body weight was monitored as an indicator of general health. For pharmacodynamic studies, tumor tissues were collected and prepared in RIPA buffer and analyzed by Western blotting. All animal experiments were carried out in accordance with guidelines of the Institutional Animal Care and Use Committee at the Shanghai Institute of Materia Medica, Chinese Academy of Sciences (Shanghai, China).

Data analysis. Data were analyzed with GraphPad Prism software (GraphPad Software, Inc., San Diego, USA). Non-linear regression analyses were carried out to generate dose–response curves and to calculate IC₅₀ values. Means ± SD were calculated automatically using this software. A paired two-tailed Student’s t-test was used to test for significance where indicated.

Results

Gastric N87-KR cells are selectively resistant to T-D1M. To investigate the molecular mechanism of T-D1M resistance, we first established T-D1M-resistant clones of the N87 gastric cancer cell line. N87 cells were grown in medium containing gradually increasing concentrations of T-D1M, ultimately yielding 14 subclones of T-D1M-resistant N87 cells (N87-KR cells), as outlined in Table 1. These N87-KR cells were strongly resistant to T-D1M, with resistance ratios ranging from 47 to 104; however, they were sensitive to trastuzumab and DM1, with resistance ratios close to 1 (Table 1, Fig. 1a). N87-KR cells were also sensitive to other HER2-targeted agents, such as HKI-272 and lapatinib, as well as the microtubule-disrupting agent, vinorelbine, and the heat shock protein 90 inhibitor, geldanamycin (data not shown). As initial studies suggested that all of these N87-KR subclones showed the same mechanism of T-D1M resistance, we focused mainly on N87-16-8 cells in the present study. Treatment with 1 µg/mL T-D1M significantly increased G2/M-phase cell-cycle arrest and levels of cleaved PARP, an apoptosis biomarker, in N87 cells, as did DM1 (Fig. 1b,c). In contrast, DM1, but not T-D1M, induced G2/M cell-cycle arrest and PARP cleavage at a concentration of 1 µg/mL in N87-16-8 cells (Fig. 1b,c). Taken together, these results indicate that N87-KR cells are selectively and strongly resistant to T-D1M.

T-D1M binding, internalization, and externalization are not involved in T-D1M resistance in N87-KR cells. T-D1M exerts its antitumor activity through several critical steps, including binding to HER2, circulating with HER2 in cells (internalization and externalization), and proteolytic degradation into the active metabolite. Factors that affect these steps may influence T-D1M activity. We determined whether these steps had been altered in N87-KR cells. We first investigated the expression of HER2, which is essential for T-D1M binding, in N87-16-8 cells. As shown in Figure 2(a), N87-16-8 cells expressed the same amount of HER2 as N87 cells. Consistent with this, both FACS analyses and confocal immunofluorescence assays indicated that T-D1M bound to N87-16-8 cells and N87 cells with an equal maximum binding rate (Fig. 2b).

Next, the circulation of the T-D1M-HER2 complex was investigated in both N87-16-8 and N87 cells. As shown in Figure 2(c), internalization of T-D1M-HER2 complexes was initially detected within 30 min and reached a plateau after 1 h, achieving the same maximum internalization rates in both N87-16-8 and N87 cells. If T-D1M externalization was investigated after internalization for 1 h, we found that the recycling curves for T-D1M in both cell lines were similar and biphasic, with initial rapid recycling followed by a platform phase after 10 min (Fig. 2d). Collectively, these results indicate that binding, internalization, and externalization of T-D1M are not involved in T-D1M resistance.

| Cell line | T-D1M | DM1 |
|-----------|-------|-----|
| N87       | 0.2 ± 0.11 (11) | 2.3 ± 0.3 (10) |
| N87-4-1   | 17.9 ± 0.2 (76) | 4.9 ± 0.6 (21) |
| N87-4-2   | 20.4 ± 0.1 (86) | 3.8 ± 0.3 (17) |
| N87-4-3   | 18.7 ± 13.4 (79) | 2.8 ± 0.8 (12) |
| N87-4-4   | 21.9 ± 7.5 (92) | 4.2 ± 0.9 (18) |
| N87-8-1   | 15.2 ± 0.1 (64) | 2.3 ± 0.3 (10) |
| N87-8-3   | 20.8 ± 9.8 (88) | 3.4 ± 0.7 (15) |
| N87-16-1  | 19.6 ± 9.6 (83) | 2.7 ± 0.6 (12) |
| N87-16-2  | 13.8 ± 2.0 (58) | 3.3 ± 0.5 (14) |
| N87-16-3  | 19.8 ± 9.0 (84) | 3.4 ± 0.4 (15) |
| N87-16-4  | 13.1 ± 0.4 (56) | 2.2 ± 0.5 (10) |
| N87-16-5  | 19.9 ± 9.1 (83) | 3.1 ± 0.0 (13) |
| N87-16-6  | 24.7 ± 0.3 (104) | 2.7 ± 0.5 (12) |
| N87-16-7  | 11.1 ± 2.6 (47) | 3.5 ± 0.3 (15) |
| N87-16-8  | 12.5 ± 0.8 (53) | 2.9 ± 1.3 (13) |

Table 1. Antiproliferative effects of trastuzumab emtansine (T-D1M) and mertansine (DM1) against T-D1M-sensitive and -resistant cell lines.

N87 and N87-KR cells were treated with different concentrations of T-D1M for 120 h. Resistance ratio = IC₅₀(R)/IC₅₀(S) where N87 and N87-KR cells were treated with different concentrations of DM1 for 72 h.
HPLC-MS. Lysine-MCC-DM1 accumulated in a time-dependent manner in both N87 and N87-16-8 cells; however, the amount of lysine-MCC-DM1 in N87 cells was approximately 1.8-fold greater than that in N87-16-8 cells after exposure to T-DM1 for 24 h (Fig. 3c). Thus, these results collectively suggest that decreases in lysine-MCC-DM1 levels are responsible...
for the inability to inhibit microtubule polymerization, leading to T-DM1 resistance in N87-KR cells.

**Aberrant V-ATPase activity contributes to the decrease in lysine-MCC-D1M in N87-KR cells.** As there were no differences in T-DM1 binding, internalization, or externalization between N87 and N87-16-8 cells, the decrease in lysine-MCC-D1M in N87-16-8 cells is highly attributable to a change in the lysosome system, in which T-DM1 is proteolytic degraded to lysine-MCC-D1M. As a proton pump that uses energy from ATP hydrolysis to produce a proton gradient, V-ATPase has been reported to play a critical role in proteolytic degradation of some systems, in which T-DM1 is proteolytically degraded to lysine-MCC-D1M. As an enzyme-cleavable linker, H-MMAE significantly inhibited microtubule polymerization and apoptosis in N87 cells (Fig. 4f, g), suggesting that V-ATPase activity is much weaker in N87-16-8 cells than in N87 cells. Collectively, these results suggest that decreased lysosomal V-ATPase activity leads to a decrease in lysine-MCC-D1M production, and that this decrease in lysine-MCC-D1M confers T-DM1 resistance in N87-16-8 cells.

**Resistance to T-DM1 is overcome by HER2 ADCs containing linkers different from that in T-DM1.** Because a metabolic disorder is the key mechanism underlying T-DM1 resistance in N87-16-8 cells, we considered using another HER2-targeted ADC containing a distinct linker as an approach to overcoming T-DM1 resistance. Accordingly, we tested H-MMAE, consisting of the HER2-targeted antibody hertuzumab and the microtubule-disrupting drug MMAE joined by an enzyme-cleavable linker, against T-DM1-resistant N87-16-8 cells. Hertuzumab-vc-MMAE exerted potent inhibitory effects on the proliferation of both N87 and N87-16-8 cells, showing equivalent IC₅₀ values (Fig. 5a). Consistent with this, H-MMAE significantly inhibited microtubule polymerization and apoptosis in N87-16-8 cells (Fig. 5b, c), suggesting that H-MMAE efficiently overcomes T-DM1 resistance in vitro.

Finally, the activities of H-MMAE and T-DM1 against N87-16-8 cells were investigated in vivo. As shown in Figure 5d, T-DM1 at a dose of 10 mg/kg inhibited the growth of N87 xenografts by 117.5%, but only inhibited N87-16-8 xenografts by 31.4%, suggesting that N87-16-8 cells are also resistant to
T-DM1 in vivo. This was confirmed by an investigation of the effects of T-DM1 on HER2 protein levels in tumor tissues, which showed that T-DM1 reduced HER2 expression only in N87 tumor tissues (Fig. 5f). These data were consistent with the in vitro results showing that T-DM1 only downregulated HER2 levels in N87 cells (data not shown). By contrast, H-MMAE at a dose of 3 mg/kg potently inhibited both N87 and N87-16-8 tumors, reducing their growth by 92.0% and 108.0%, respectively. Moreover, H-MMAE reduced the expression of HER2 in both N87 and N87-16-8 tumor tissues (Fig. 5f). Taken together, these results indicate that H-MMAE efficiently overcomes T-DM1 resistance both in vitro and in vivo.

Discussion

Antibody–drug conjugates are emerging as a powerful class of antitumor agents. (26,27) However, drug resistance inevitably
develops following long-term treatment. Preclinical models can provide valuable tools for predicting likely mechanisms of resistance to these drugs, enabling drug-resistance mechanisms in the clinic to be identified, understood, and eventually overcome. We therefore modeled acquired resistance to T-DM1 using HER2-overexpressing gastric cancer N87 cells following chronic exposure to gradually increasing amounts of T-DM1. Few mechanisms of T-DM1 resistance have been reported. Here, we show for the first time that reducing the levels of the active T-DM1 metabolite, through decreased lysosomal V-ATPase activity, confers resistance to T-DM1. Notably, this resistance can be overcome by H-MMAE, another HER2-targeted ADC with an enzyme-cleavable linker.

Trastuzumab emtansine inhibits the growth of HER2-positive cancer cells through the actions of its two components: trastuzumab and DM1. Resistance to trastuzumab has been a major concern for years, and a number of possible mechanisms have been reported. These include increased epidermal growth factor receptor and HER3 expression, reactivation of HER3 signaling, increased mucin 4 expression, and upregulation of insulin-like growth factor-1 receptor, activation of SRC or c-Met, a reduction in phosphatase and tensin homolog and expression of P-glycoprotein, or MDR-associated protein. Some resistance mechanisms reported for microtubule disrupting agents include overexpression of P-glycoprotein, or MDR-associated protein and breast cancer resistance protein, mutation of tubulin, and overexpression of βIII-tubulin. Some resistance mechanisms for HER2-targeted ADCs have also been reported. Using in vitro T-DM1 resistance models from the HER2-overexpressing esophageal adenocarcinoma cell line OE-19, Sauveur and colleagues found that resistant cells became less sensitive to trastuzumab, and a subpopulation among the resistant cells showed increased expression of MDR1. Loganzo and colleagues reported that increased levels of the drug-efflux protein ABCB1 in 361-TM cells and decreased HER2 levels in JIMT1-TM cells were responsible for mediating resistance to trastuzumab–maytansinoid, similar results were reported by Lewis Phillips. It would appear to be self-evident that the resistance mechanisms of HER2-targeted ADCs primarily reflect resistance to the HER2-targeted antibody or the conjugated chemotherapeutic agent. In the current study, we revealed a novel resistance mechanism of HER2-targeted ADCs, showing that T-DM1 resistance in N87-KR cells is not contributed by trastuzumab or DM1, because N87-KR cells were sensitive to trastuzumab and DM1, as well as other HER2-targeted agents and microtubule-disrupting agents. Instead, T-DM1 resistance in N87-KR cells resulted from a T-DM1 metabolic disorder caused by reduced V-ATPase activity in lysosomes. Using an N87-TM cell drug-resistant model, Sung and colleagues reported findings that are congruent with our results. However, unlike our results, they reported that ADCs are internalized into caveolin 1-positive puncta, altering their trafficking to the lysosome and leading to T-DM1 resistance. Interestingly, we also found that hertuzumab–vc-MMAE, another HER2-targeted ADC containing a cleavable linker, efficiently overcame T-DM1 resistance induced by metabolite reduction. Thus, the linker used in ADCs, which is typically neglected, should be accorded greater attention during the design of ADC structures.

Lysosomes provide a suitable pH and various proteolytic enzymes for the degradation of macromolecules, including some kinds of ADCs. Erickson and their coworkers have shown that lysosomal processing is required for the activity of antibody-maytansinoid conjugates, showing that Baf-A1, a selective V-ATPase inhibitor, almost completely abolished G2/M cell-cycle arrest induced by huC242-SMCC-DM1. It has also been reported that many drug-resistant cell lines have a lower intracellular pH than their drug-sensitive counterparts.

![Image](https://www.wileyonlinelibrary.com/journal/cas)
A decrease in cytotoxic effects owing to passive ion trapping-based lysosomal sequestration and an increase in the number of drug-accumulating lysosomes has been shown to enhance chemoresistance. The data presented in the current study confirmed that a suitably acidified environment is essential for T-DM1 metabolism. Importantly, the N87-KR cell line established here showed dramatically reduced acidification. Thus, V-ATPase, which produces a proton gradient for proteolytic degradation of T-DM1, plays a critical role in the antitumor activity of T-DM1. A decrease in V-ATPase activity may inevitably disrupt T-DM1 metabolism and ultimately lead to T-DM1 resistance, and the sole approach for overcoming this resistance is to find other drugs to replace T-DM1. Therefore, we propose that V-ATPase activity may serve as a biomarker for T-DM1 resistance. If a substantial reduction in V-ATPase activity is detected, T-DM1 is not suitable for use.

In conclusion, we reported a novel mechanism of T-DM1 resistance in which a decrease in the level of the active metabolite mediates T-DM1 resistance in HER2-positive gastric cancer cells. We propose that V-ATPase activity in lysosomes is a novel biomarker for predicting T-DM1 resistance and further suggest that HER2-targeted ADCs containing a
Aberrant metabolism confers T-DM1 resistance

![Graph showing inhibition percentage vs Log [H-MMAE] (ng/mL)]

- N87
- N87-16-8

IC₅₀ (ng/mL)

- N87: 9.6 ± 0.2
- N87-16-8: 9.2 ± 0.0

![Western blot images](b)

- T-DM1 (10 μg/mL)
- H-MMAE (1 μg/mL)

- PARP
- Cleaved PARP
- GAPDH

![Western blot images](c)

- Polymeric tubulin
- Polymeric actin

![Graphs showing mean tumor volume SEM (mm³) and mean body weight (g)]

- Vehicle
- H-MMAE 3 mg/kg
- T-DM1 10 mg/kg

![Graphs showing mean tumor volume SEM (mm³) and mean body weight (g)]

- Vehicle
- H-MMAE 3 mg/kg
- T-DM1 10 mg/kg

![Western blot images](f)

- Control
- T-DM1
- H-MMAE

- HER2
- Tubulin

- HER2
- Tubulin
protease-cleavable linker are capable of overcoming T-DM1 resistance induced by the reduction in V-ATPase activity.

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Disclosure Statement
The authors have no conflict of interests.

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