DS-8201a, a new HER2-targeting antibody–drug conjugate incorporating a novel DNA topoisomerase I inhibitor, overcomes HER2-positive gastric cancer T-DM1 resistance

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Anti-HER2 therapies are beneficial for patients with HER2-positive breast or gastric cancer. T-DM1 is a HER2-targeting antibody–drug conjugate (ADC) comprising the antibody trastuzumab, a linker, and the tubulin inhibitor DM1. Although effective in treating advanced breast cancer, all patients eventually develop T-DM1 resistance. DS-8201a is a new ADC incorporating an anti-HER2 antibody, a newly developed, enzymatically cleavable peptide linker, and a novel, potent, exatecan-derivative topoisomerase I inhibitor (DXd). DS-8201a has a drug-to-antibody-ratio (DAR) of 8, which is higher than that of T-DM1 (3.5). Owing to these unique characteristics and unlike T-DM1, DS-8201a is effective against cancers with low-HER2 expression. In the present work, T-DM1-resistant cells (N87-TDMR), established using the HER2-positive gastric cancer line NCI-N87 and continuous T-DM1 exposure, were shown to be susceptible to DS-8201a. The ATP-binding cassette (ABC) transporters ABCC2 and ABCG2 were upregulated in N87-TDMR cells, but HER2 overexpression was retained. Furthermore, inhibition of ABCC2 and ABCG2 by MK571 restored T-DM1 sensitivity. Therefore, resistance to T-DM1 is caused by efflux of its payload DM1, due to aberrant expression of ABC transporters. In contrast to DM1, DXd payload of DS-8201a inhibited the growth of N87-TDMR cells *in vitro*. This suggests that either DXd may be a poor substrate of ABCC2 and ABCG2 in comparison to DM1, or the high DAR of DS-8201a relative to T-DM1 compensates for increased efflux. Notably, N87-TDMR xenograft tumor growth was prevented by DS-8201a. In conclusion, the efficacy of DS-8201a as a treatment for patients with T-DM1-resistant breast or gastric cancer merits investigation.

HER2, a member of the human epidermal growth factor receptor family, is aberrantly expressed in certain malignancies, including breast and gastric cancer, primarily due to HER2 genomic amplification.[1] Dysregulated HER2 expression is associated with increased risk of recurrence and poorer prognosis in these cancers.[2–7] In HER2-positive malignancies, this protein can stimulate downstream RAS-RAF-ERK and PI3K-PTEN-AKT signaling, and play a key role in cell proliferation.[8] Therefore, HER2 is the preferred therapeutic target in such cancers. For example, anti-HER2 monoclonal antibodies such as trastuzumab and pertuzumab and HER2 tyrosine kinase inhibitors such as lapatinib have been shown to improve survival, and are used as standard therapies for HER2-positive cancers, including those of the breast and stomach.[9–12]

Antibody–drug conjugates (ADCs) comprising an anti-HER2 monoclonal antibody, a linker and a cytotoxic agent payload are also employed as a targeted therapy for HER2-positive malignancies. The ADC T-DM1, which consists of the anti-HER2 monoclonal antibody trastuzumab conjugated to the tubulin polymerization inhibitor DM1, is already in use for the treatment of HER2-positive breast cancer.[13] In its phase III clinical trial (the EMILIA study), T-DM1 was found to improve the survival and quality of life of HER2-positive breast cancer patients.[14] Furthermore, 44% of patients responded to T-DM1 treatment, although its efficacy was limited in other cases. A better understanding of the mechanism underlying this limitation could improve the treatment of patients with HER2-positive cancer.

What's new?

In certain cancers, amplification and dysregulation of the human epidermal growth factor receptor HER2 is associated with more aggressive disease and worse prognosis, making it an appealing therapeutic target. Of special clinical interest in this regard is the antibody–drug conjugate (ADC) DS-8201a, which consists of an anti-HER2 antibody, a peptide linker, and an exatecan-derivative topoisomerase I inhibitor (DXd). In the current preclinical study with gastric cancer cells, DS-8201a was found to overcome resistance to T-DM1, an established HER2-targeting ADC, by upregulating the ATP-binding cassette trans-porters ABCC2 and ABCG2. Moreover, the DS-8201a Dxd payload successfully inhibited the growth of T-DMI-resistant cells.

DS-8201a, another HER2 ADC, is composed of a humanized anti-HER2 antibody, a newly developed, enzymatically cleavable peptide linker, and an exatecan-derivative topoisomerase I inhibitor (DXd).[15–18] This unique linker-payload system reduces the hydrophobicity of the ADC.[15] Thus, DS-8201a can carry eight molecules of DXd per antibody, whereas the drug-to-antibody ratio (DAR) of other ADCs currently ranges from 2 to 4.[15,19] These unique characteristics of DS-8201a contribute to its preclinical efficacy against T-DM1-insensitive tumors with low HER2 expression.[15,18] DS-8201a is therefore viewed as a promising treatment for patients with breast or gastric cancer expressing low HER2 levels (immunohistochemistry [IHC]1+ and IHC2+/FISH−).

Although relative to T-DM1, DS-8201a has been shown to be effective against cancers with low HER2 expression, its effect on HER2-positive malignancies that have acquired resistance to T-DM1 after a durable response is unclear. The aim of this study was to evaluate the efficacy of DS-8201a using an HER2-positive gastric cancer cell line in which resistance to T-DM1 had been induced, and to investigate the mechanisms underlying the differences in effectiveness between these ADCs.

Material and Methods

Cells and reagents

HER2-amplified NCI-N87 gastric cancer cells (N87-parent) were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS and 1% penicillin–streptomycin. This medium contained no biologic ligands, including epidermal growth factor (EGF). T-DM1 and trastuzumab were obtained from commercial sources. DS-8201a, Dxd and DM1 were provided by Daiichi Sankyo Co., Ltd (Tokyo, Japan).

Generation of T-DM1-resistant (TDMR) cells

N87-parent cells were initially exposed to 0.1 μg/mL T-DM1 and subsequently cultured in the presence of gradually increasing doses, up to a maximum of 4 μg/mL. Once established, the resistant cell line, designated N87-TDMR, was maintained in medium containing the maximal dose of T-DM1 (4 μg/mL) to maintain selective pressure for T-DM1 resistance.

In vitro growth inhibition assay

Cells were plated in 96-well flat-bottomed plates at 1.0×10^4 (N87-parent) and 1.5×10^4 (N87-TDMR) cells per well in RPMI 1640 medium containing 2% FBS. After incubation for 24 hr, T-DM1, trastuzumab, DM1, and DXd were added at a range of concentrations. Following further incubation for 72 hr, cell viability was assessed with the use of a CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA). Luminescence values are expressed as percentages of that observed for untreated cells, and the IC50 of each drug was calculated.

In vitro proliferation assay

The cell proliferation assay was performed by plating 3.0×10^5 N87-parent cells and 1.0×10^6 N87-TDMR cells in RPMI 1640 medium containing 10% FBS, 1% penicillin–streptomycin and T-DM1 or DS-8201a onto a 150-mm² tissue culture dish. Every 5 days, the medium was changed, the number of cells was counted with a hemocytometer, and cell viability was measured by trypan blue exclusion. The experiment was repeated three times.

Antibodies and western blotting

Cells were seeded at 1×10^6 cells per plate and allowed to grow overnight in medium containing 2% FBS, before being harvested. Western blotting was carried out as previously described.[20] Proteins were transferred to nitrocellulose membranes, which were probed with the following antibodies: phospho-EGF receptor (EGFR; Tyr1068), EGFR and phospho-HER3 (Tyr1289) (all from Cell Signaling Technology, Danvers, MA, USA); β-actin (Sigma-Aldrich); and phospho-HER2 (Tyr1248) and HER2 (both Merck/Millipore, Darmstadt, Germany).

Phosphoreceptor tyrosine kinase (RTK) array

Cells were lysed with NP40 lysis buffer following incubation for 24 hr in RPMI 1640 medium supplemented with 0.1% FBS. Cell lysates were centrifuged at 14,000g for 5 min. Supernatants were then incubated with the Human Phospho-RTK Array (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

IHC assessment of HER2 expression

Sections of formalin-fixed, paraffin-embedded tumor tissue (4 μm thick) were placed on slides coated with polylysine. After deparaffinization and blocking of endogenous peroxidase, HER2 immunostaining was performed using rabbit anti-human c-erbB-2 primary antibody, diluted 1:100 (Dako Corp., Carpinteria, CA, USA). Primary antibody binding was assessed using the Dako Quick-Staining, Labeled Streptavidin-Biotin System (Dako Corp.) and subsequent addition of diaminobenzidine as a chromogen.[21] Scoring was performed according to the clinical practice guideline for breast cancer.[22]

Reverse transcription and real-time (RT) PCR analysis

One microgram of total RNA from the cultured cell lines was reverse transcribed to cDNA using a GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA, USA). RT-PCR was performed using SYBR *Premix Ex Taq* and the Thermal Cycler Dice system (TaKaRa, Kusatsu, Japan), as described previously.[23] The experiment was repeated three times.

Microarray

Microarray analysis was carried out using the GeneChip Human Transcriptome Array (HTA) 2.0 (Affymetrix, Santa Clara, CA, USA). Following the Affymetrix recommended protocol, cRNA was prepared from 100 ng total RNA and used to generate ssDNA, which was fragmented and biotinylated. Labeled ssDNA was hybridized for 16–18 hr at 45°C on HTAs, which were then washed and stained with a streptavidin–phycoerythrin conjugate in an Affymetrix Fluidics Station 450 instrument. The microarrays were scanned with a GeneChip Scanner 3000 7 G (Affymetrix) according to manufacturer guidelines. The CEL files generated were analyzed with Affymetrix Expression Console Software (version 1.4), which normalizes array signals using a robust multiarray averaging algorithm. The normalized data were then further analyzed using Transcriptome Analysis Console 3.0 software (Affymetrix).

In vivo tumor growth inhibition assay

All animal experiments were performed in accordance with the Recommendations for Handling of Laboratory Animals for Biomedical Research compiled by the Committee on Safety and Ethical Handling Regulations or Laboratory Animal Experiments, Kindai University. The study was also reviewed and approved by the Animal Ethics Committee of Kindai University. N87-parent or N87-TDMR cells (5×10^6 per mouse) were subcutaneously injected into the right flanks of female BALB/cAJcl-*nu/nu* mice (CLEA Japan, Tokyo, Japan). Once tumors had reached the target volume (0.2 cm^3), mice were randomly assigned to treatment and control groups. On Day 0, mice received a single intraperitoneal injection of PBS (100 μL; as control), T-DM1 (10 mg per kg body weight in 100 μL PBS) or DS-8201a (10 mg per kg body weight in 100 μL PBS). Tumor volumes and mouse body weights were measured twice per week. Mice were sacrificed if tumors became necrotic or grew to a volume of 2.0 cm^3. Tumor volume was defined as $1/2 \times$ length $\times$ width^2.

Statistical analyses

Statistical analyses were performed using SPSS version 22.0 (IBM Corp., Armonk, NY, USA). All statistical tests were two-sided, unpaired t-tests and p values <0.05 were considered statistically significant. Data were graphically depicted using GraphPad Prism 5.0 for Windows (GraphPad Software, Inc., La Jolla, CA, USA).

Results

N87-TDMR cells retain the HER2 expression level of N87-parent cells

We first generated a T-DM1-resistant cell line by continuous exposure of NCI-N87 gastric cancer cells to gradually increasing concentrations of T-DM1 over a 6-month period. These N87-TDMR cells proliferated freely in 4 μg/mL T-DM1. Furthermore, an *in vitro* growth inhibition assay confirmed that they were no longer susceptible to T-DM1 (Fig. 1a). Specifically, the viability of N87-parent cells decreased according to the concentration of T-DM1 in the medium after 3 days of exposure, whereas N87-TDMR cells proliferated even at the highest T-DM1 dose (10 μg/mL). The T-DM1 IC50 was found to be 0.55 and >10 μg/mL for N87-parent and N87-TDMR cells, respectively (Fig. 1a). A growth inhibition assay using trastuzumab revealed the limited susceptibility of both N87-TDMR and N87-parent cells to this antibody when treated alone (Fig. 1b), implying that DM1, the payload of T-DM1, played a critical role in preventing proliferation of N87-parent cells treated with this ADC. Indeed, N87-parent cells proved to be susceptible to DM1, and more so than N87-TDMR cells. The DM1 IC50 was 0.046 μM for the former, and >1 μM for the latter (Fig. 1c).

As ADCs are highly dependent on antigen expression to exert their targeted cytotoxic effects, we measured HER2 levels to investigate the mechanisms underlying T-DM1 resistance. Immunoblotting showed that total and phosphorylated HER2 expression remained high in N87-TDMR cells, with similar levels observed in N87-parent cells (Fig. 1d). Furthermore, in xenograft tumor specimens, HER2 was found to be overexpressed using the N87-TDMR and N87-parent models, both of which had an HER2 IHC score of 3+ (Fig. 1e), consistent with our *in vitro* observation. We also evaluated other RTKs, as their dysregulation has been shown to cause drug resistance in previous studies.[24,25] Levels of EGFR, HER3 and their phosphorylated forms were similar between N87-parent and N87-TDMR cells (Fig. 1d). Moreover, the phospho-RTK array demonstrated that phosphorylation of other RTK types, such as c-Met and insulin-like growth factor 1 receptor, was comparable in N87-parent and N87-TDMR cells (Supporting Information, Fig. S1).
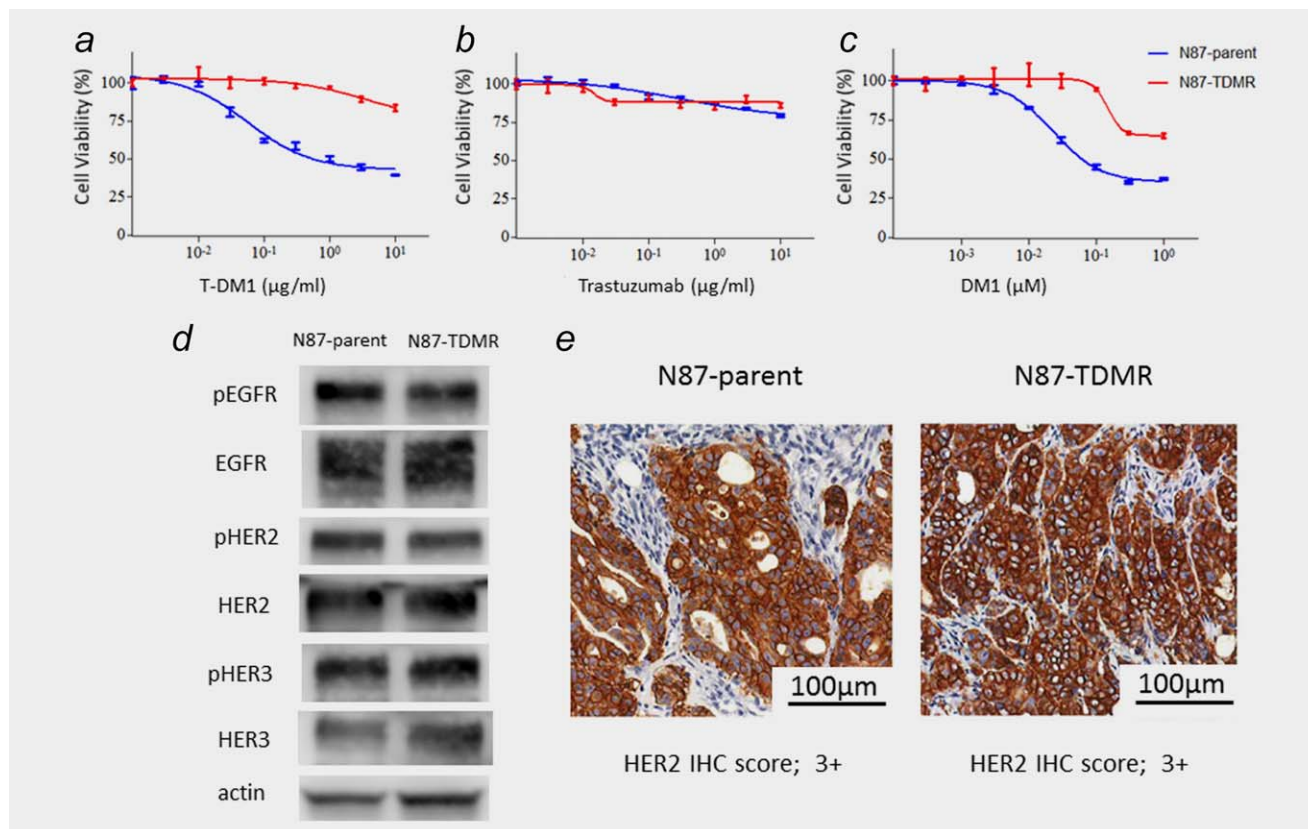
Figure 1. (*a–c*) N87-parent and N87-TDMR cells were treated with T-DM1 (*a*), trastuzumab (*b*) or DM1 (*c*) at the indicated concentrations. Cell viability (shown as means $\pm$ SDs, $n = 6$) was measured after 72 hr of treatment and plotted relative to that of the untreated group. (*d*) N87-parent and N87-TDMR cell lysates were subjected to immunoblotting to detect the indicated proteins. (*e*) IHC detection of HER2 in mouse xenograft tumors derived from N87-parent and N87-TDMR cells. The HER2 IHC score of each sample is shown.

Together, these results suggest that resistance to T-DM1 by N87-TDMR cells was not caused by loss of expression of its target, HER2.

Aberrant expression of ATP-binding cassette (ABC) transporters mediates T-DM1 resistance in N87-TDMR cells

Next, we screened for transcripts correlated with T-DM1 resistance by comprehensively comparing gene expression in N87-TDMR and N87-parent cells. Expression of HER2 and components of its signaling pathway was not markedly altered in N87-TDMR cells, although levels of the ABC transporters ABCC2 and ABCG2 were increased compared with N87-parent cells (Table 1). Aberrant expression of ABCC2 and ABCG2 in N87-TDMR cells was confirmed by RT-PCR (59.2 and 22.4 times greater, respectively, than that observed in N87-parent cells, Fig. 2*a*).

For a functional evaluation of ABCC2 and ABCG2 in N87-TDMR cells, we used MK571, an inhibitor of these two proteins. Although N87-TDMR cells were able to proliferate when exposed to T-DM1, even at the highest concentration of 10 μg/mL, their viability decreased with increasing doses of this ADC when treated together with 100 μM MK571 (Fig. 2*b*). A combination of 10 μg/mL T-DM1 and 100 μM MK571 significantly decreased the number of viable N87-

TDMR cells compared to treatment with each agent alone (Fig. 2*c*).

These observations imply that T-DM1 resistance in N87-TDMR cells depended principally on aberrant expression of ABCC2 and ABCG2.

DS-8201a inhibits the proliferation of N87-TDMR cells

We subsequently examined the effect of DS-8201a on N87-TDMR cells, and compared it to that of T-DM1. N87-parent and N87-TDMR cells were left untreated or cultured in the presence of 0.2 μg/mL T-DM1 or DS-8201a for 15 days. N87-parent cells failed to proliferate over the course of the experiment when treated with either ADC (Fig. 3*a*). In contrast, N87-TDMR cells were able to proliferate when exposed to T-DM1, although they proved to be susceptible to DS-8201a. The number of viable N87-TDMR cells was significantly lower in the DS-8201a group after 10 days (Fig. 3*a*).

We also tested the susceptibility of N87-parent and N87-TDMR cells to DXd, the payload of DS-8201a. Cells were incubated with 0.3 μM DM1 (the T-DM1 payload) or DXd for 3 days. N87-parent cells were susceptible to both DM1 and DXd, with the proportion of viable cells being ∼25% of that in the control group (Fig. 3*b*). The proliferation of N87-TDMR cells was similar under control and DM1 treatments;

Table 1. Differentially expressed mRNAs in N87-parent and N87-TDMR cells detected by microarray

Gene symbol	Fold change (linear)
ABC transporters	
ABCC2	**50.2**
ABCC3	−1.5
ABCG2	**8.0**
ABCB1	1.2
RTKs	
EGFR	3.2
HER2	−1.5
HER3	1.3
HER4	1.0
c-Met	2.4
IGF1R	1.4
HER3 ligands, NRGs	
NRG1	−1.3
NRG2	−1.2
NRG3	1.1
NRG4	−11.1
HER2 downstream pathway	
KRAS	−1.2
NRAS	−1.1
BRAF	1.0
PIK3CA	−1.7
PTEN	1.7
MTOR	3.6
SRC	1.8
ADCs trafficking factors	
CAV1	−3.1
PTRF	−2.5

Abbreviations: ABC, ATP-binding cassette; RTK, receptor-type tyrosine kinase; EGFR, epidermal growth factor receptor; NRG, neuregulin; PI3K, phosphoinositol 3 kinase; CAV1, caveolae protein 1; PTRF, polymerase I and transcript release factor.

however, they were susceptible to DXd, which resulted in the number of viable cells being <50% of that in the control group.

Thus, the different susceptibility of N87-TDMR cells to T-DM1 and DS-8201a may have been due to the distinct payloads of each of these ADCs.

In vivo antitumor activity of DS-8201a versus T-DM1

The stability of a linker-payload system may be compromised in plasma.[26] Therefore, in addition to our *in vitro* investigation, we evaluated the efficacy of DS-8201a in treating an N87-TDMR xenograft tumor mouse model, and compared it to that of T-DM1. Both ADCs effectively restricted the growth of the xenograft tumors generated from N87-parent

cells over the 5 weeks of the experiment. In contrast, tumors derived from N87-TDMR cells grew during this period in spite of T-DM1 treatment (Fig. 4*a*); however, their volumes were reduced in mice administered DS-8201a.

ADC payload release in plasma can cause toxicity. However, although the antitumor effects of DS-8201a were more potent, body weights in this group were no different from those of control mice (Fig. 4*b*). These findings indicate that DS-8201a can overcome T-DM1 resistance in a xenograft model without inducing toxicity.

Discussion

In this study, we report for the first time that NCI-N87 gastric cancer cells with acquired resistance to T-DM1 remain susceptible to DS-8201a, an alternative HER2 ADC. Although T-DM1 is now a standard therapy for HER2-positive breast cancer, some patients do not respond to T-DM1.[14] In addition, in a large, randomized clinical trial, T-DM1 did not improve the survival of patients with HER2-positive gastric cancer.[27] This leaves ample scope to improve HER2 ADCs for the treatment of HER2-positive cancer. Our results suggest that DS-8201a has the potential to ameliorate the clinical outcome of malignancies resistant to T-DM1. Consistent with our observations, others have reported that HER2-positive gastric cancer (HER2 3+, FISH+) patient-derived xenograft (PDX) tumors are also susceptible to DS-8201a, in spite of being resistant to T-DM1.[15] Unfortunately, the mechanism responsible for T-DM1 resistance in this PDX model was unknown, meaning that the processes underlying the differences in susceptibility to these two agents could not be explained. In addition to these preclinical data, Tamura *et al.* reported the results of a dose escalation study forming part of a phase I trial of DS-8201a and its efficacy.[28] Preliminary efficacy results indicated an objective response rate of 35% among 16 breast cancer and five gastric cancer patients. Specifically, of 12 patients with HER2-positive breast cancer previously treated with T-DM1 and with *de novo* or acquired resistance to this ADC, five achieved partial response after DS-8201a administration. Together, these data demonstrate that DS-8201a may be able to overcome resistance to T-DM1 in HER2-positive gastric or breast cancer.

N87-TDMR cells expressed significantly higher levels of the ABC transporters ABCC2 (also known as MRP2) and ABCG2 (also known as BCRP), which mediate the efflux of drugs from cells. Furthermore, an inhibitor of these transporters, MK571, restored the susceptibility of N87-TDMR cells to T-DM1. Similarly, in a previous study, T-DM1-resistant breast cancer cells were shown to upregulate ABCB1 (also known as P-glycoprotein or MDR1), of which DM1 is a substrate.[29,30] Thus, DM1 efflux by such proteins is thought to be responsible for T-DM1 resistance. More generally, ABC transporters may reduce the effectiveness of diverse small-molecule chemotherapeutic agents. As N87-TDMR cells remained susceptible to DXd, the payload of DS-8201a, this molecule may represent a poor substrate for ABCC2 and
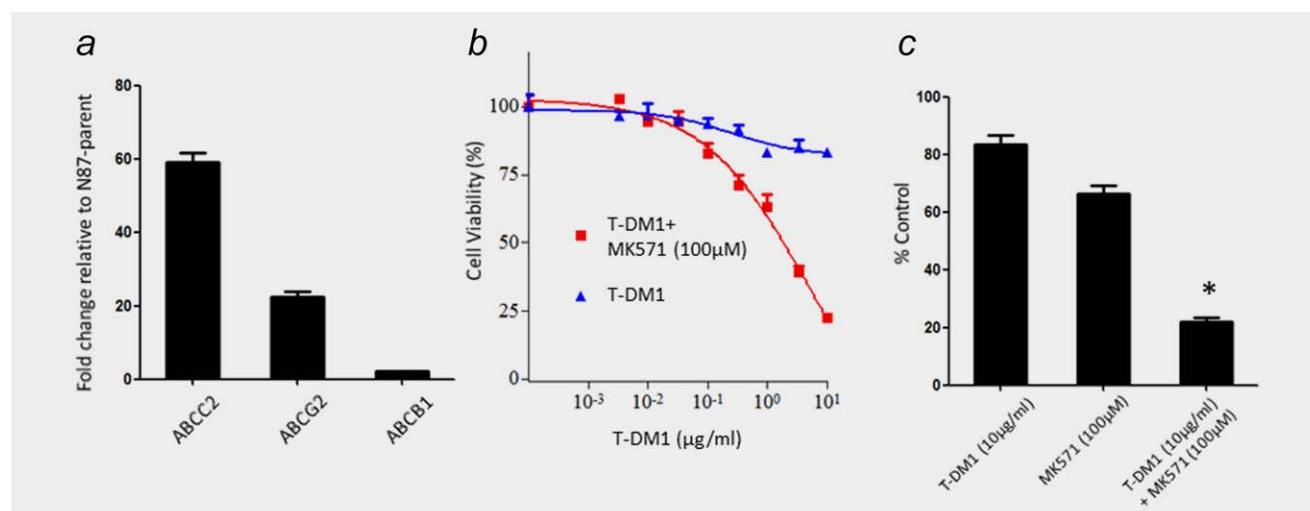
Figure 2. (*a*) mRNA expression levels (means ± SDs, *n* = 3) of ABCC2, ABCG2 and ABCB1 in N87-TDMR cells relative to N87-parent cells, as determined by RT-PCR. (*b*) N87-TDMR cells were treated with T-DM1 alone, MK571 alone or both drugs at the indicated concentrations. Cell viability (shown as means ± SDs, *n* = 6) was measured after 3 days of treatment. (*c*) N87-TDMR cells were treated with T-DM1 (10 μg/mL) or MK571 (100 μM) alone or in combination. Cell viability (shown as means ± SDs, *n* = 6) was measured after 72 hr of treatment and plotted relative to that of the untreated group. *$p < 0.05$ compared to T-DM1 alone and MK571 alone.
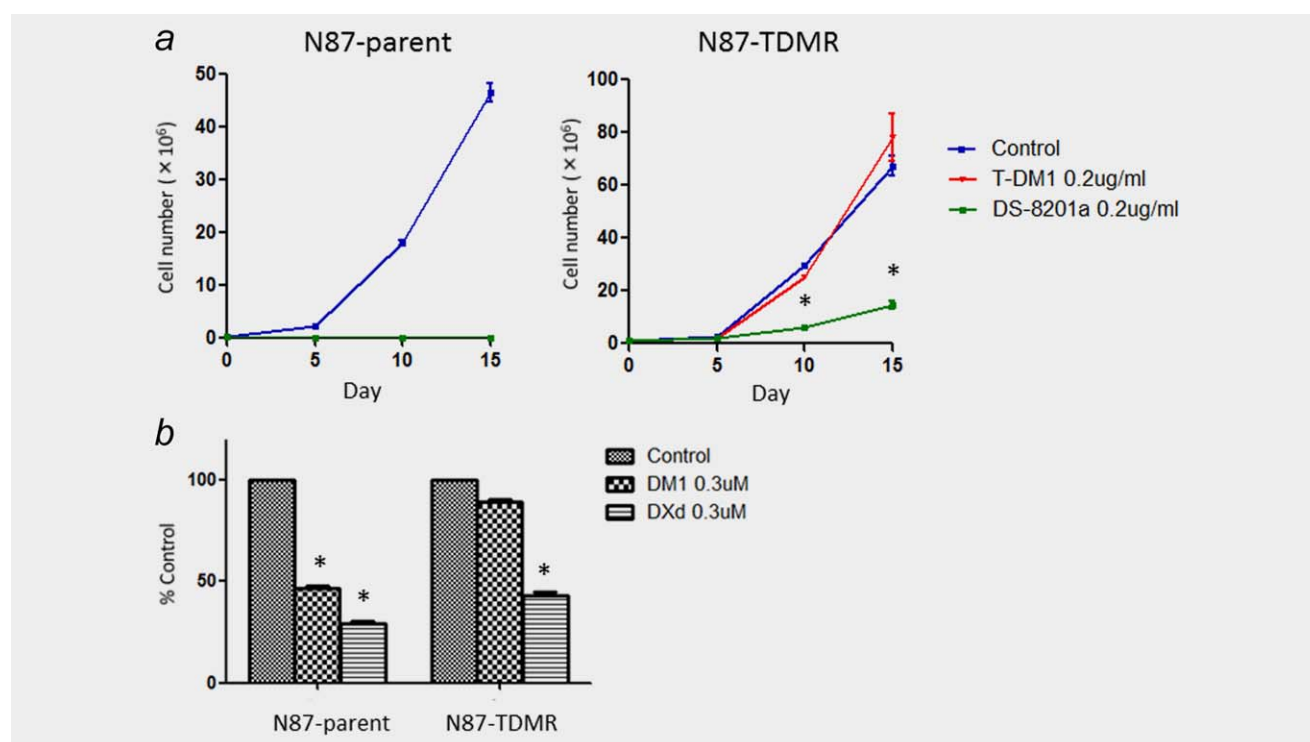

Figure 3. (*a*) Proliferation of N87-parent and N87-TDMR cells treated with T-DM1 or DS-8201a. Cells were left untreated or treated with T-DM1 (0.2 μg/mL) or DS-8201a (0.2 μg/mL) every 5 days for 15 days. The number of cells was counted on days 5, 10, and 15 with a hemocytometer. Cell viability was measured by trypan blue exclusion. The *y*-axis shows the cell number per well, and the values depicted are means ± SDs of three independent experiments in which each sample was processed in triplicate. *$p < 0.05$ compared to the control and T-DM1. (*b*) N87-parent and N87-TDMR cells were left untreated or administered DM1 (0.3 μM) or DXd (0.3 μM). Cell viability (shown as means ± SD, *n* = 6) was measured after 72 hr of treatment and plotted relative to that of the control group. *$p < 0.05$ compared to the control.

ABCG2 compared to DM1. Cross-resistance to T-DM1 and DS-8201a does not appear to occur, owing to the different transporters implicated in the excretion of their payloads from the cell.

Alternatively, the fact that the structure of DS-8201a results in a DAR of 8 might contribute to its potent effect on tumors with acquired resistance to T-DM1. In general, the
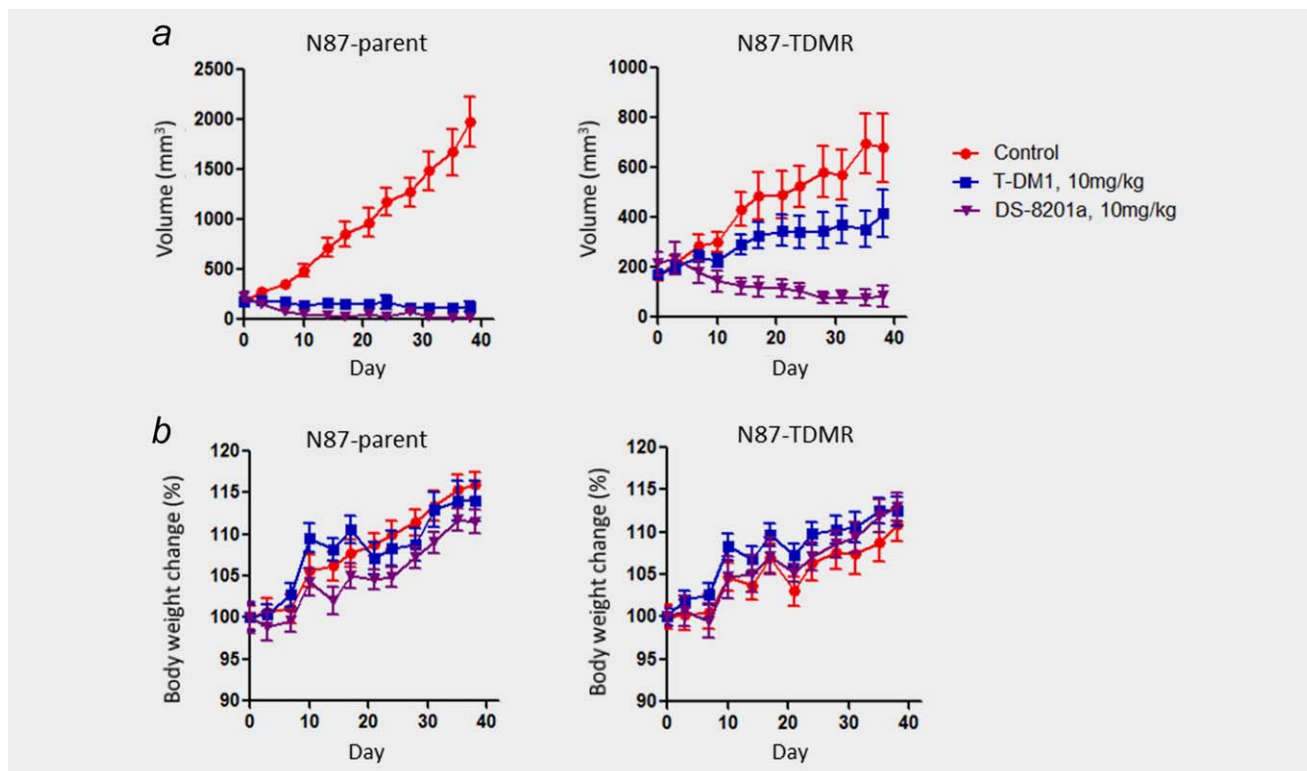
Figure 4. Volumes (*a*) of xenograft tumors derived from N87-parent or N87-TDMR cells, and body weight change (*b*) of the host mice after treatment with T-DM1, DS-8201a or PBS (control). Values shown are means ± SDs (*n* = 10).

DARs of ADCs are limited (that of T-DM1 is 3.5) because greater DARs tend to result in unstable complexes with higher clearance rates, which can limit efficacy and increase toxicity. The newly developed linker system employed in DS-8201a reduces the hydrophobicity of this ADC and increases its DAR. Therefore, DS-8201a is stable in plasma, demonstrates slower clearance, and achieves greater intracellular delivery of its payload to cancer cells.[15] Kovtun *et al.* replaced the *N*-succinimidyl-4-(maleimidomethyl)cyclohexane-1-carboxylate linker of T-DM1 with a more hydrophilic PEG_4Mal linker, and showed the resulting conjugates to have improved potency *in vitro* and *in vivo* in MDR1-expressing tumor models.[31] Furthermore, Loganzo *et al.* reported that switching a noncleavable linker for the protease-cleavable mcValCit-PABC chain could effectively overcome acquired T-DM1 resistance in at least two cell models.[30,32] These results demonstrate that the unique linker of DS-8201a may contribute to the potent efficacy of this conjugate against T-DM1-resistant cancer.

Antigen loss can cause resistance to ADCs, although in the present work, N87-TDMR cells aberrantly expressed HER2 to the same degree as N87-parent cells. Loganzo *et al.*

subjected cells of the HER2-positive breast cancer line JIMT1 to cycles of high-dose T-DM1, inducing resistance to this ADC *in vitro*.[30] These JIMT1-TM-resistant cells exhibited a marked decrease in HER2 protein expression after several months of T-DM1 exposure. The discrepancy between N87-TDMR and JIMT1-TM cells in this respect might be explained by cell-dependent differences or variations in the methods used to establish resistance. Various other mechanisms have also been proposed to explain T-DM1 resistance, including modulation of trafficking.[24–26,29,30,32] Although the processes behind T-DM1 resistance are heterogeneous, DS-8201a may be effective in treating resistant cells, through a mechanisms that depends on HER2 expression and drug efflux by ABC transporters. Furthermore, those molecular alterations were not completely elucidated in T-DM1-resistant tumors. Therefore, molecular characterization of tumors may further acknowledge the superiority of DS-8201a compared to T-DM1.

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