Title

Defective cyclin B1 induction in trastuzumab-emtansine (T-DM1) acquired resistance in HER2-positive breast cancer

Running title

Cyclin B1 and T-DM1 acquired resistance

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Conflict of interest

A.L.L. and J.A. have participated in advisory boards and in the speakers bureau from Roche. No potential conflicts of interest were disclosed by the other authors.

Statement of significance

We report a preclinical link between T-DM1 resistance and failure to induce cyclin B1 in breast cancer. This finding may be of translational relevance in terms of incorporation of a pharmacodynamic assay to test cyclin B1 induction in breast cancer patients treated with T-DM1 as potential early biomarkers of responding patients.
TRASLATIONAL RELEVANCE

Trastuzumab-emtansine (T-DM1) is an antibody-drug conjugate constituted by trastuzumab linked to DM1 (a tubulin polymerization inhibitor). T-DM1 is a standard treatment in advanced HER2 positive metastatic breast cancer. However, resistance inevitably occurs. To date, no clinical biomarker of T-DM1 resistance has been identified. In this study we obtained three T-DM1 acquired resistant breast cancer \textit{in vitro} models. We report that cyclin B1 induction is a hallmark of T-DM1 activity in both trastuzumab primary sensitive and resistant cells. In HER2 positive breast cancer cells with acquired T-DM1 resistance, the drug failed to induce cyclin B1, while reducing cyclin B1 degradation in these cells partially restores sensitivity. In fresh human HER2 positive breast cancer explants, the induction (but not the baseline levels) of cyclin B1 by T-DM1 correlated with apoptosis, suggesting that a pharmacodynamic assay to test cyclin B1 induction in breast cancer patients treated with T-DM1 may help to identify early the patients more likely to benefit from that drug treatment.
ABSTRACT

Purpose: Trastuzumab-emtansine (T-DM1) is a standard treatment in advanced HER2 positive breast cancer. However, resistance inevitably occurs. We aimed to identify mechanisms of acquired T-DM1 resistance.

Experimental Design: HER2-positive breast cancer cells (HCC1954, HCC1419, SKBR3 and BT474) were treated in a pulse-fashion with T-DM1 to induce a resistant phenotype. Cellular and molecular effects of T-DM1 in parental versus resistant cells were compared. CDK1 kinase activity and cyclin B1 expression were assayed under various conditions. Genetic modifications to up or down regulate cyclin B1 were conducted. Effects of T-DM1 on cyclin B1 levels, proliferation and apoptosis were assayed in human HER2 positive breast cancer explants.

Results: We obtained three cell lines with different levels of acquired T-DM1 resistance (HCC1954/TDR, HCC1419/TDR and SKBR3/TDR cells). HER2 remained amplified in the resistant cells. Binding to HER2 and intracellular uptake of T-DM1 were maintained in resistant cells. T-DM1 induced cyclin B1 accumulation in sensitive but not resistant cells. Cyclin B1 knock-down by siRNA in parental cells induced T-DM1 resistance, while increased levels of cyclin B1 by silencing cdc20, partially sensitized resistant cells. In a series of 18 HER2-positive breast cancer fresh explants, T-DM1 effects on proliferation and apoptosis paralleled cyclin B1 accumulation.

Conclusion: Defective cyclin B1 induction by T-DM1 mediates acquired resistance in HER2 positive breast cancer cells. These results support the testing of cyclin B1 induction upon T-DM1 treatment as a pharmacodynamic predictor in HER2 positive breast cancer.

Key words: Trastuzumab-emtansine (T-DM1), acquired resistance, cyclin B1, HER2 positive breast
INTRODUCTION

Trastuzumab-emtansine (T-DM1) is an antibody drug-conjugate (ADC) consisting of the anti-HER2 antibody trastuzumab covalently linked to the antimitotic agent DM1 through a stable linker that potently inhibits growth of both trastuzumab-sensitive and -resistant HER2-amplified cancer cells (1). T-DM1 has mechanisms of action containing of the anti-tumor effects related to trastuzumab and those associated with intracellular DM1 catabolites (2,3). DM1 is a derivative of maytansine, a highly potent antimitotic drug (4). Once bound to HER2, T-DM1 enters in the cell by receptor-mediated endocytosis and the HER2-T-DM1 complex is processed via degradation of trastuzumab in lysosomes giving rise to the intracellular release of the active catabolite Lys-MCC-DM1. In the cytoplasm, DM1 exerts its functions through binding to the beta subunit of tubulin and modifying its assembly properties. By doing so, DM1 is able to disrupt the formation of the mitotic spindle necessary for accurate chromosome segregation along mitotic process. Overall, DM1 as other antimitotic drugs elicits a mitotic arrest and cells therefore fail to complete a normal mitosis. This prolonged cell cycle delay eventually culminates in cell death by mitotic catastrophe, necrosis or apoptosis (5). In addition to these DM1-related actions, T-DM1 maintains properties of trastuzumab such as inhibition of HER2-directed signal transduction and activation of antibody-dependent cell-mediated cytotoxicity (2,3).

T-DM1 is a standard second-line treatment for HER2 positive metastatic breast cancer patients based on the results of a phase III clinical trial (EMILIA trial), that compared the efficacy and toxicity of T-DM1 versus the combination of lapatinib and capecitabine in patients previously treated with trastuzumab and chemotherapy. T-DM1 offered superior activity, tolerability and survival than lapatinib and capecitabine (6). However, resistance to T-DM1 occurs (3,7,8). To date, the main T-DM1 clinical resistance mechanisms studies included an exposure-response and exploratory biomarker analysis, both based on patients from the EMILIA and TH3RESA trial (9-11). In the exposure-response analysis, the data showed that higher T-DM1 exposure was associated with improved efficacy. This analysis suggested that there is an opportunity to optimize T-DM1 dose in the patient subgroup with low exposure (10). With regards to the exploratory biomarker study of the
EMILIA trial, focusing only in the T-DM1 group, univariate analysis of the data suggested that there were no evident differences in progression free survival (PFS) related to EGFR, and HER3 median mRNA concentration ratios, PIK3CA mutations status or PTEN expression; patients with HER2 mRNA above the median had better outcomes on T-DM1 than those equal or below the median (11). In the TH3RESA phase III trial, performed in heavily pre-treated HER2-positive advanced breast cancer, patients were randomized to T-DM1 or treatment of physician's choice. An exploratory biomarker analysis included HER2 and HER3 mRNA expression, PIK3CA mutation status and PTEN protein expression. T-DM1 prolonged median PFS in all the subgroups analyzed. A numerically greater benefit was reported in patients with tumors expressing HER2 mRNA above the median (9). More recently, in the ZEPHIR trial, the use of molecular imaging of HER2 by HER2-PET/CT with (89)Zr-trastuzumab combined with early metabolic response assessment by FDG-PET/CT, discriminated patients with short versus long time to T-DM1 treatment failure (12).

The biomarker analyses discussed above were focused on the HER2 signaling pathway. However, given the dual mechanism of action of T-DM1, we focused on resistance potentially related to the cell cycle modulating effects of DM1. We identified that the G2/M arrest induced by T-DM1 in sensitive HER2 breast cancer cells did not occur in their resistant counterparts in a CDK1/cyclin B1 dependent manner. In fresh HER2 positive breast cancer explants, lack of induction of cyclin B1 correlated with T-DM1 failure to induce apoptosis.
MATERIAL AND METHODS

Cell lines and reagents. Breast cancer cell lines BT474, SKBR3, AU565, EFM-192A, HCC1954 and HCC1419 were obtained from the American Type Culture Collection. Authenticity of the cells was tested by STR DNA Profiling analysis at the ATCC (June 2013 and December 2014) before starting the generation of resistant cells. The number of passages between thawing and use in the described experiments was five or less. T-DM1 (trastuzumab emtansine, Kadcyla) was provided by Genentech under MTA agreement (Sliwkowski MX, Lewis Phillips GD) and trastuzumab by Hospital del Mar pharmacy.

Cell proliferation assays. Cells were plated in duplicate into 12-well culture plates at a density of 8-12×10^3 cells per well and left overnight and then on day zero, treatment was initiated. At this time T-DM1 [0.1-1 µg/mL] was added. On days 3, 7 and 10, cells were washed with PBS, trypsinized, resuspended in media and counted with Scepter Automated Cell Counter (Millipore).

Generation of cell lines with acquired T-DM1 resistance. T-DM1-resistant cell lines were derived from original parental cell lines by exposure to stepwise increasing concentrations of T-DM1 in a pulse-fashion (13). The protocol is summarized in Figure 1A. Three T-DM1 acquired-resistant sublines were collected named SKBR3/TDR, HCC1954/TDR and HCC1419/TDR. Additionally, vehicle treated parental cell lines were kept in culture during this period as control cell lines. We established an exposure to 0.1 µg/mL of T-DM1 for 3 days as a reference schedule to define resistance in our model. This schedule was selected based on experiments performed in MCF7 cells that do not overexpress HER2, whose growth was unaffected until higher concentrations and longer exposure to T-DM1 were used.

Fluorescent in situ hybridization (FISH) for HER2. Formalin-fixed and paraffin-embedded cell pellets were prepared from parental and T-DM1 resistant cells to assess the status of HER2 gene by scoring its amplification following ASCP/CAP guidelines (14). The commercial PathVysion HER2 DNA probe was used.
**T-DM1/HER2 receptor binding.** T-DM1 binding to the cell surface was evaluated by indirect immunofluorescence staining and flow cytometry. Cells (2-5×10⁵) were incubated with T-DM1 (7.5 μg/mL) for 30 min followed by R-Phycoerythrin AffiniPure F(ab')₂ Fragment Goat Anti-Human IgG, Fcγ Fragment Specific (Jackson ImmunoResearch; West Grove, PA, USA) (1:500 dilution); for 30 min, at 4ºC. Trastuzumab (7.5 μg/mL) and Rituximab (MabTera, Roche; 7.5 μg/mL) were used as positive and negative controls, respectively. Live/dead gate was set with DAPI counterstaining. Samples were acquired on LSR Fortessa flow cytometer (BD Biosciences), and data analyzed with FlowJo software (TreeStar).

**T-DM1 internalization assay.** T-DM1 internalization was evaluated by immunofluorescence staining. Cells (1.5 x 10⁵) were seeded on coverslips and treated with 1.5 μg/mL T-DM1 for 15 minutes. After washing out the drug, cells were cultured for 24 hours with or without 5 μM chloroquine (a drug that induce changes in lysosomal pH) to accumulate intracellularly T-DM1. Cy3-conjugated anti-human was used to detect T-DM1, phalloidin-FITC (P5282 Sigma) was used for actin staining and nuclei were counterstained with DAPI. Sample processing was performed as previously reported (15).

**Cell cycle assay.** Cells were seeded on 6-well plates and treated with compounds for 24 hours by T-DM1 [0.1μg/mL]. Thereafter, cells were incubated with 30 μM bromodeoxyuridine (BrdU, B9285, Sigma) for 30 min at 37°C, and then subsequently trypsinized, washed with PBS, and fixed in ice-cold ethanol for at least one hour at -20°C. Cells were digested in pre-warmed 1 mg/mL pepsin in 30 mM HCl (pH 1.5) for 30 min at 37°C with gentle shaking, and then incubated in 2 M HCl for 20 min at room temperature. Next, cells were washed with PBS and antibody buffer (0.5% w/v BSA, 0.5% v/v Tween-20 in PBS) and incubated with mouse primary antibody against BrdU (555627, BD Pharmingen) in antibody buffer for one hour at RT. After washing with PBS, samples were incubated with secondary goat anti-mouse IgG FITC-conjugated antibody for 30 min at room temperature in the dark. Finally, cells were washed one more time with PBS and then stained with 25 μg/mL propidium iodide. Flow cytometry was performed using a Becton Dickinson FACScan operated by the CELLQuest software.
Cdc2/CDK1 activity assay. Cdc2/CDK1 kinase activity was measured with the nonradioactive MESACUP® Cdc2/CDK1 Kinase Assay Kit (MBL, International Corporation) following the manufacturer instructions. The method is based on an ELISA assay that utilizes a biotinylated synthetic peptide as a substrate for the Cdc2/CDK1 kinases present in the samples and a monoclonal antibody conjugated to horseradish peroxidase recognizing the phosphorylated form of the peptide. The horseradish peroxidase substrate is then added and the intensity of the color was measured at 492 nm. The results are reported as fold-induction of OD 492 nm in arbitrary units (RLA) of treated sample using untreated condition as reference set at 1.

Immunocytochemistry (ICC). Cells were seeded on glass coverslips and cultured as indicated in the figure legends. Cells washed by PBS, and then fixed in 4% paraformaldehyde for 10 min at room temperature. Then the samples permeabilized by PBS containing 0.25% Triton X-100, and then, incubated with 1% BSA in PBST for 30 mins for blocking unspecific binding of the antibodies. In the next step, samples incubated for 1 hour with anti-α tubulin FITC conjugated antibody (F2168, Sigma). Cell nuclei were stained with DAPI (1:1000; Sigma) for 15 min at room temperature. After washing, coverslips were mounted with a drop of mounting medium and viewed using a Leica SP5 upright confocal microscope.

Immunoblotting. Cellular protein lysates were prepared in lysis buffer [50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1% (v/v) Nonidet P-40, 0.1% (w/v) SDS] containing protease inhibitors and quantified by use of the Bradford assay (Bio-Rad Laboratories). Equivalent protein amounts of each sample were analyzed. Protein detection on Western blots was performed according to standard protocols. The antibodies used were: HER2 (Biogenex), cyclin B1 (sc-245) and CDC2 p34 (CDK1) (sc-54) purchased from SantaCruz and β-actin (A-5316) was purchased from Sigma.

Apoptosis and cell death analysis. For measuring apoptosis, the Annexin V and Dead Cell Assay Kit (Millipore) was used according to the manufacturer's instructions. Briefly, after treatment, the cells were incubated with Annexin V and Dead Cell Reagent (7-AAD) for 20 min at room temperature in the dark, and the events for dead, late apoptotic, early
apoptotic, and live cells were counted with the Muse Cell Analyzer (Millipore, Hayward, CA, USA) and analyzed with MuseSoft 1.4.0.0 (Millipore).

**Cyclin B1 and cdc20 silencing.** For transient transfection experiments, cells were transfected by use of the Amaza 4D-Nucleofector™ device, according to manufacturer's instruction. For each electroporation reaction, 100 μl of complete Nucleofector® solution combined with 300 nM siRNA against Cyclin B1, cdc20 and scrambled siRNA as a control (duplex siRNAs were purchased from GE Dharmacon) under a specific optimized program for siRNA delivery with the Nucleofector (SKBR3 E-009).

**Exposure of fresh human breast cancer explants to T-DM1 ex vivo.** The study was approved by the ethics committee of the Hospital del Mar and conducted following institutional guidelines. Fresh tumor specimens from women with HER2 positive breast cancer undergoing routine cancer surgery, which were not needed for diagnostic purposes, were collected to add ex vivo T-DM1 and assess its molecular effects according to our experience (16). Samples were sliced and cultured in RPMI 1640 medium supplemented with 10% FBS, 2mM L-glutamine and 100 U/mL penicillin-streptomycin for 120 hours in the absence (control) or presence of T-DM1 [0.1μg/mL]. Specimens were fixed in 10% neutral-buffered formalin for 16 hours and embedded in paraffin then assayed by immunohistochemistry.

**Immunohistochemistry (IHC).** Three-micrometer-thick paraffin sections from tissue blocks of the tumors were stained for HER2 (Herceptest P980018/S010, Dako), cyclin B1(sc-245, Santacruz), Ki-67 (GA62661-2, MIB1 clone, Dako), phosphorylated (Ser10) Histone 3 (9701, Cell Signaling) and Caspase-3 active (9664, Cell Signaling) followed by incubation with an anti-rabbit Ig dextran polymer (Flex+, Dako) and 3,3’-diaminobenzidine as chromogen in a Dako Link platform. HER2 staining was scored following ASCP/CAP guidelines (14). For the other markers, the percentage of positive tumor cells was scored.

**Statistical analysis.** Data are presented as mean ± Standard Deviation (SD). The Graph Pad Prism® software was used to construct graphs and statistical analysis. Statistical
significance was determined using student's t test or One-Way ANOVA. P value ≤ 0.05 was considered as significant.
RESULTS

Generation of T-DM1 resistant HER2-positive breast cancer cells

We assessed the half maximal inhibitory concentration (EC50) values of T-DM1 in a panel of HER2 positive breast cancer cells. The median EC50 values for BT474, SKBR3, AU565, EFM-192A, HCC1954 and HCC1419 cell lines at 72 hours were 0.025, 0.002, 0.005, 0.009, 0.020 and 0.018 µg/mL, respectively. Four cell lines, two sensitive to trastuzumab (SKBR3 and BT474) and two with primary resistance to trastuzumab (HCC1954 and HCC1419) were selected to generate T-DM1 resistance (TDR) by applying a pulsed administration strategy of the drug, often used for the development of chemotherapy drug-resistance (Figure 1A) (13). This protocol encompasses short pulses of drug treatment followed by rest periods in drug-free media to allow the cells to recover from toxicity between treatments until a stable resistant phenotype is observed. Specifically, the procedure consisted of three consecutive cycles of 3 days on treatment followed by 3 days off treatment for each T-DM1 concentration of 1, 2 and 4µg/mL. The entire 54-day protocol resulted in the generation of cells with varying levels of T-DM1 resistance.

We established an exposure to 0.1 µg/mL of T-DM1 for 3 days as a reference schedule to define resistance in our model. This schedule was selected based on experiments performed in MCF7 cells that do not overexpress HER2, whose growth was unaffected until higher concentrations and longer exposure to T-DM1 were used. After approximately two months, three cell lines developed resistance to T-DM1 (called HCC1954/TDR, HCC1419/TDR and SKBR3/TDR-Figure 1B). We were unsuccessful to generate BT474 resistant cells even after repeated attempts. HCC1954/TDR cells were completely resistant after 10 days of exposure to T-DM1 at 0.1µg/mL. HCC1419/TDR and SKBR3/TDR cells decreased the sensitivity to the antiproliferative effects of T-DM1 by 20-30% at 0.1µg/mL after 3 days. This level of resistance, albeit modest, has been helpful to identify mechanisms of resistance to several agents (13). T-DM1 resistant cells had a growth rate similar to parental cells in vitro, as assessed by cell counting after 7 days of culture under the same conditions. The relative cell numbers of resistant versus parental cells were 110% ± 8.3 in HCC1954/TDR, 93% ± 4 in HCC1419/TDR and 103% ± 7 in SKBR3 (no
The morphology of parental versus resistant cells was similar by optic microscopic observation. All resistant cell lines exhibited resistance features for at least one year after their generation. Of note, SKBR3/TDR cells retained a similar antiproliferative response to trastuzumab alone than parental cells (50% growth inhibition), suggesting that the resistance was associated to DM1 (Figure 1C). Both parental and resistant cells showed similar EC50 values for paclitaxel ((HCC1954 (4.5nM), HCC1954/TDR (6.5nM), HCC1419 (6.0nM), HCC1419/TDR (5.6nM), SKBR3 (3.2nM), SKBR3/TDR (3.1nM)).

**T-DM1 binds to cell surface HER2 and is internalized in resistant cells**

HCC1419/TDR and SKBR3/TDR cells retained the same level of HER2 amplification and protein expression than parental cells (Figure 2A and 2B). However, HCC1954/TDR cells displayed decreased amplification of HER2 and reduced HER2 protein (Figure 2A and 2B), and mRNA levels (6% of relative mRNA expression) compared with parental cells. Parental HCC1954 cells had a mean of 40 HER2 copies but they contained two additional subpopulations; 17% of the cells had less than 6 copies (some scored as amplified for having only one copy of CEP17) and 11.4% had 6-10 HER2 copies. During the generation of the resistant cells, T-DM1 eradicated the cells with strong HER2 amplification after a short exposure, allowing the emergence of sub-dominant clones with low HER2 amplification (average number of 8 HER2 copies, HER2 1+ by immunohistochemistry). The emergence of HER2 low resistant cells in HCC1954 was confirmed in three independent experiments. Despite this important change in the population, the resistant cells still met the criteria for scoring them as HER2 amplified since they have more than 6 HER2 copies (14,17).

Additionally, other genes such as ORMDL3, STARD3, PPP1R1B, MIEN1 located in the minimal common region of 17q12 amplification were also down modulated as assayed by expression arrays. SKBR3/TDR and HCC1419/TDR did not have any significant differences in the levels of HER2, ORMDL3, STARD3, PPP1R1B and MIEN1 mRNA in comparison to parental cells, as assayed by qRT-PCR. We next assayed the ability of T-DM1 to bind to cell surface HER2 by flow cytometric analysis. Although surface HER2 levels might vary in
HCC1954/TDR compared with parental cells, T-DM1-HER2 binding was preserved in all TDR cells (Figure 2C).

We assayed whether T-DM1 was capable of inducing internalization upon binding to HER2 in paired sensitive and resistant cells (15). Cells were treated with 10 nM T-DM1 for 15 minutes at 37°C. After washing out the drug, cells were cultured for 24 hours with or without 50 μM chloroquine to accumulate T-DM1 intracellularly. As shown in Figure 2D, T-DM1 (red dots) was internalized into both parental and resistant cells. The magnitude of T-DM1 internalization and intracellular pattern were similar in parental and resistant SKBR3 and HCC1419 cells. In HCC1954/TDR cells we also detected T-DM1 intracellularly, but to a lesser extent than in their parental HER2 positive counterpart, in agreement with their lower HER2 amplification level and the surface expression.

Effects of T-DM1 on G2/M arrest and mitotic catastrophe in parental vs resistant cells

T-DM1 increased significantly the percentage of cells in G2/M phase while it induced a decrease in the S and G0/G1 phases in the three parental cell lines (Figure 3A). In the resistant cells, the effects of T-DM1 on G2/M cell cycle arrest were less pronounced than in parental cells. In HCC1954/TDR and HCC1419/TDR there were no increase in G2/M. In SKBR3 there was a significant increase following T-DM1 but to a much lesser degree than in parental cells (Figure 3A). Next, we evaluated the effects of T-DM1 on microtubule arrangement. In parental cells T-DM1 caused the formation of multi-nucleated giant cells with severely defective microtubule arrangements (Figure 3B). Previously it has been reported that T-DM1 causes tumor growth inhibition by mitotic catastrophe (18). These morphological alterations suggestive of mitotic catastrophe were undetected in T-DM1 resistant cells. Since most cells undergoing mitotic catastrophe are destined to die by apoptosis, we evaluated the effects of T-DM1 on apoptosis induction. T-DM1 induced apoptosis in HCC1954 and SKBR3 parental cells at 48 hours but the effect on the resistant counterparts was much less pronounced under the same conditions (Figure 3C).
T-DM1 resistant cells failed to induce CDK1/cyclin B1

We hypothesized that resistant cells may have a defective cell cycle regulatory machinery that does not allow T-DM1 to induce G2/M arrest and consequently, mitotic catastrophe. We focused on the potential involvement of Cyclin Dependent Kinase 1 (CDK1) and cyclin B1 in T-DM1 resistance. Activation of the CDK1-cyclin B1 complexes are essential for progression into M-phase, but prolonged mitotic arrest (for example because of the inability of forming the mitotic spindle in cells treated with microtubule-affecting agents such as DM1) leads to mitotic catastrophe (19). The activity of CDK1-cyclin B1 complex is regulated mainly by the expression of cyclin B1 and by the phosphorylation status of the catalytic subunit CDK1. Degradation of cyclin B1 by the proteasome after ubiquitination by the multi-subunit ubiquitin E3-ligase APC/C^cyc20^ is essential for exiting mitosis. T-DM1 exposure augmented cyclin B1 expression in a panel of HER2 positive parental breast cancer cells (except BT-474) (Figure 4A).

We analyzed the effects of T-DM1 on the activity of CDK1. We first tested this effect in four parental HER2 positive cell lines and in their trastuzumab resistant (TR) counterparts that we recently reported (20). These TR cell lines were included in this analysis to confirm whether acquired trastuzumab resistance (that corresponds to the clinical setting in which T-DM1 is approved) affects T-DM1 ability to induce CDK1 activity. The results showed a significant increase in CDK1 Kinase activity 24 hours post-treatment with T-DM1 [0.1 µg/mL] in both sensitive and trastuzumab resistant cells by using the MESACUP® Cdc2/Cdk1 Kinase Assay Kit with exception of the BT-474 cell pairs (Figure 4B). In all ll the cell lines with acquired trastuzumab resistance T-DM1 exposure decreased viable cells (cell number relative to control at 3 days of 20% ± 3.2 in AU565/TR, 40% ± 6 in EFM-192A, 17% ± 3 in SKBR3/TR and 72 % ± 3 in BT474/TR) and cyclin B1 was induced following drug exposure (Figure 4B). Trastuzumab alone did not induce cyclin B1 in these cell lines (Figure 4C).

We then assessed T-DM1 effects on the CDK1/cyclin B1 complex kinase activity and cyclin B1 levels in the paired cell lines parental and resistant to T-DM1. We performed a time course study the HCC1954 and HCC1954/TDR cells. The activation of the mitotic kinase
CDK1/cyclin B1 was detected as early as 12 hours post-T-DM1 in parental HCC1954 cells (2 fold) but not in HCC1954/TDR cells (p<0.05). Protein levels of total CDK1 were unchanged in parental and resistant cells. Cyclin B1 protein levels were moderately increased (2 fold) at 12 hours following T-DM1 treatment in parental cells in parallel with CDK1 activity, and they were maintained elevated up to 24 hours afterwards. This increase in the cyclin B1 levels was undetected in resistant cells (Figure 4D). A similar pattern of cyclin B1 expression and appearance of CDK1/cyclin B1 kinase activity were observed with the other T-DM1 sensitive/resistant cell pairs after 24 hours of T-DM1 exposure (Figure 4C). The analysis of the levels of cyclin B1 mRNA by qRT-PCR showed a non-significant increase (Supplementary Figure 1), suggesting posttranscriptional regulatory mechanisms.

**Cyclin B1 mediates T-DM1 resistance**

We tested whether cyclin B1 knock-down could mediate resistance to T-DM1 in parental cells. Cell lines were transfected with siRNA directed against cyclin B1 mRNA or with a suitable control. Cyclin B1 levels were measured 24 and 48 hours after the transfection by western blot confirming that they were significantly lower in the siRNA transfected cells than in the siControl (Figure 5A). The paired cell lines transfected with the cyclin B1 siRNA or the siControl were exposed to T-DM1 and cell viability was assayed after 48 hours. Silencing of cyclin B1 induced a significant resistance to T-DM1 in the three parental cell lines (Figure 5A).

We next tested whether increasing the levels of cyclin B1 in resistant cells might sensitize them to T-DM1. Cdc20 is a regulatory subunit of the multi-subunit ubiquitin E3-ligase APC/C that is responsible of cyclin B1 degradation at the end of mitosis. We silenced cdc20 to promote cyclin B1 accumulation (21). As shown in Figure 5B, cyclin B1 protein level were increased following cdc20 silencing in 2 out of the three resistant cells. In HCC1954/TDR cells, no differences in T-DM1 sensitivity between control (scrambled siRNA) and cdc20-silenced cells were observed. However, T-DM1 resistance was partially reverted in cdc20 silenced SKBR3/TDR and HCC1419/TDR cells (Figure 5B).
Induction of cyclin B1 by T-DM1 in HER2 positive human breast cancer explants associates with apoptosis

To overcome, at least in part, the issue of the limited number of cell lines, we tested the association between cyclin B1 induction and T-DM1 antiproliferative effect in a panel of 18 fresh human HER2 positive breast cancer explants. The results confirmed such association. We assayed the basal levels of cyclin B1 in 18 HER2 positive breast cancers according to our experience in fresh explants (16). In 7 cases, cyclin B1 was undetected and in the remaining 11, the percentage of tumor cells with detected cyclin B1 staining ranged from 2% to 10% (Figure 6A). A fraction of these tissues were cultured ex vivo for 5 days in the presence of T-DM1 or control and assayed for modulation in cyclin B1 expression. There were two main observations; first, cyclin B1 was induced in the majority of explants exposed to T-DM1 (12 out of 18, 66.6% of the tumors), including those with undetected baseline levels; second, the percentage of tumor cells that stained positive for cyclin B1 was dramatically increased in many specimens (61.1% of cases), a finding consistent with the induction of a G2/M arrest by T-DM1 (Figure 6A and 6B). These findings support the effect of T-DM1 on inducing a persistent accumulation of cyclin B1 in tumor, which was a hallmark of T-DM1 effects in our panel of sensitive breast cancer cells. Of note, in 4 explants with cyclin B1 detected at baseline, T-DM1 exposure did not further increase the percentage of expressing cells. We also assayed induction of apoptosis by expression of Caspase-3 active, proliferation (Ki67) and activation of the M-phase checkpoint kinases (phospho-Histone H3, p-H3) (Figure 6A). With regards to proliferation, Ki67 staining was not modulated by T-DM1 after 5 days while reduction in p-H3 staining paralleled the induction of apoptosis by T-DM1. The lack of an effect on Ki67 is consistent with the fact that cells arrested in G2/M also stain positive to Ki67 (Figure 6A) (22). We found two main patterns of cyclin B1 and apoptosis changes following T-DM1 exposure: no/very low cyclin B1 accumulation and very low up-regulation of apoptosis versus strong cyclin B1 (accumulation) and high induction of apoptosis. Trastuzumab alone did not induce cyclin B1 in ex vivo explants (Figure 6B). The tumor cell areas with cyclin B1 staining and the ones
with Caspase-3 active staining did not frankly overlapped in the tissue sections, suggesting
different stages of T-DM1 effects.

Fifteen explants were from diagnostic specimens derived from patients that received
neoadjuvant treatment without T-DM1. The other three were from metastatic patients.
Two received T-DM1 and had cyclin B1 and apoptosis induction ex vivo. One had de novo
metastatic disease, the explant was from the diagnostic breast cancer biopsy, and
received T-DM1 as second line achieving a partial response. The second patient had bone
and liver disease and after several lines of treatment received T-DM1. The explant was
obtained from a liver metastasis just before T-DM1 (Supplementary Figure 2) and
subsequently had a partial response.
DISCUSSION

We have generated three different HER2-positive breast cancer cell lines with various levels of acquired T-DM1 resistance by applying a pulsed administration strategy of the drug, an approach commonly used for the development of chemotherapy drug-resistant cancer cells (13). The resistant phenotype was achieved within the first two months of drug exposure, suggesting the emergence of a chemotherapy-driven mechanism. We chose initially four cell lines to develop resistance, but in one of them (BT474) we were unsuccessful. The three remaining cell lines, albeit not sufficient to establish the generalizability of the findings, were sufficient to test mechanistically the role of cyclin B1. Similarly, the degree of acquired resistance varied in the 3 cell lines but a role of cyclin B1 in T-DM1 resistance was observed in all of them. The modest resistance observed in two of the cell lines clearly suggested that cyclin B1-independent mechanisms of action of T-DM1 remain active.

The generation of resistant cell lines in a short timeframe may be caused by several mechanisms and may vary between cell lines. In HCC1954, a specific finding was a marked reduction of HER2 gene amplification after the first round of exposure to T-DM1. In parental HCC1954 cells there was a predominant subpopulation (~93 % of cells) with high Her2 amplification and a minority subpopulation (~7 %) with low, but amplified HER2 gene. An early clonal selection of the subpopulation with lower HER2 amplification following T-DM1 exposure appears to contribute to resistance. Regardless of this, the rapid emergence of resistance, also in cell lines that retain the same level of HER2 amplification, suggests a mechanistic link with the cytotoxic DM1 component rather than to trastuzumab, by as yet unknown mechanisms such as epigenetic and/or cellular pathway rewiring. SKBR3 T-DM1 resistant cells retained sensitivity to trastuzumab, whereas the other two remained trastuzumab resistant. This suggests that multiple mechanisms of resistance may coexist in the same cell line. Additional studies are needed to understand if these mechanisms coexist in a single cell or whether this represents tumor cell heterogeneity (as in HCC1954 relative to HER2 amplification). Nevertheless, the
anti-mitotic essence of DM1 should be considered as a source of heterogeneity generation.

The cytotoxic effect of T-DM1 might be impaired by inefficient internalization or enhanced percentage of HER2-T-DM1 complex that is recycled back to the cell surface (3). It is believed that the HER2/T-DM1 conjugate enters cancer cells via the clathrin-dependent endocytosis pathway. However, a clathrin-independent mechanism, such as caveolae membranes composed mainly by caveolin-1 has also been demonstrated (23). Furthermore, it has been shown that the high endocytic activity of stem cell-like breast cancer cells make them particularly sensitive to T-DM1 (24). We have shown that T-DM1 was internalized and exhibited a similar intracellular pattern in parental and resistant cells, albeit HCC1954/TDR cells had less T-DM1 detected intracellularly. Overall, it appeared that the pathway mediating HER2-T-DM1 endocytosis was intact in resistant cell lines.

In parental cells, T-DM1 induced G2-M arrest and mitotic catastrophe. The phenomenon of ‘mitotic catastrophe’ considered a mode of cell death per se resulting from prolonged mitotic arrest is now believed to represent a pre-stage of apoptosis or even necrosis or senescence (25). In two of the three cell lines sensitive to T-DM1, the mitotic arrest induced by T-DM1 preceded apoptosis. Mitotic catastrophe did not occur in resistant cells suggesting that the machinery for inducing cell cycle arrest at the G2-M phase was disrupted.

This led us to hypothesize a potential involvement of CDK1 and cyclin B1, the members of mitotic promoting factor complex, in T-DM1 resistance (19). Entry into mitosis is initiated by CDK1 and binding of CDK1 to cyclin B1 is essential for its activation. CDK1/cyclin B1 complex and the kinase activity of CDK1 is controlled by cyclin B1 accumulation. During mitosis, chromosome segregation is facilitated by the kinetochore, an assembly of proteins built on centromeric DNA that attach chromosomes to spindle microtubules. After the last unattached kinetochore is attached to microtubules and the chromosomes are properly aligned, the mitotic checkpoint is switched off. CDK1 is then inactivated as cyclin B is rapidly degraded, and cells progress through anaphase, undergo cytokinesis and exit mitosis (26). However, in the presence of an anti-mitotic agents (such as DM1), the
impossibility of assembling the mitotic spindle activates the mitotic checkpoint arresting
the cells in mitosis for a prolonged period and they may undergo mitotic catastrophe
followed by cell death (27).

In the three parental HER2 positive breast cancer cells, cyclin B1 induction and
CDK1/cyclin B1 activation were observed following T-DM1 treatment and were
maintained elevated for up to 24 hours. However, T-DM1 failed to raise cyclin B1 levels
and, consequently, CDK1/Cyclin B1 activity in T-DM1 resistant cells. Furthermore, silencing
of cyclin B1 induced resistance to T-DM1 in parental cell lines while increasing the levels of
cyclin B1 in resistant cells sensitized them to T-DM1 in two out of the three cell lines. The
failure to generate T-DM1 BT474 resistant cells under our experimental protocol may be
related, at least in part, to the inability of T-DM1 to upregulate cyclin B1.

Other CDK-cyclin complexes have been previously reported as implicated in resistance to
anti-HER2 therapies. For instance, cyclin E has a role in trastuzumab resistance and
treatment with CDK2 inhibitors has been proposed for tumors displaying cyclin E
amplification/overexpression (28,29). A relationship of CDK4/cyclin D1 activity in
resistance to trastuzumab and lapatinib has been also reported and high levels of
cyclin D1 predicted poor response to trastuzumab (30). Preclinical studies have also shown
that residual cells surviving within 48 hours following T-DM1 treatment began to re-enter
the cell cycle and the sequential treatment with CDK4/6 inhibitors suppressed the
proliferation of these residual/resistant clones (31). A clinical trial with the CDK4/6
inhibitor palbociclib in combination with T-DM1 (NCT01976169) in HER2 positive patients
is underway. On the other hand, our results showing that CDK1/Cyclin B1 activity is
needed for T-DM1 action suggests a note of caution regarding possible combinations of T-
DM1 with pan-CDKs inhibitors or selective CDK1 inhibitors (32).

In human breast cancer, cyclin B1 expression has been associated with poor survival (33)
and is a prognostic proliferation marker in lymph node negative breast cancer cohorts
(34). In our series of fresh HER2 positive breast tumor explants the basal levels of cyclin B1
were not significantly related to T-DM1 apoptotic or antiproliferative effects. Instead, we
found that the induction of cyclin B1 following T-DM1 exposure ex vivo was highly associated to induction of apoptosis and reduced tumor cell proliferation.

Interestingly, two patients with metastatic breast cancer had a response to T-DM1 and in both of them cyclin B1 was upregulated by T-DM1 ex vivo. These anecdotal data suggest that cyclin B1 induction in explants may be associated to clinical response. On the other hand, the lack of a predictive effect of baseline cyclin B1 expression is in line with an observation in 7 HER2 positive breast cancer patients that had been treated at Hospital del Mar with T-DM1 as part of a neoadjuvant clinical trial. Four of them had detectable levels of cyclin B1, of whom 3 achieved a pathological complete response (pCR). In the 3 patients with undetected baseline cyclin B1, one pCR was achieved. Albeit patients received additional anti-HER2 agents or systemic chemotherapy as part of their neoadjuvant treatment, this few cases suggest that baseline cyclin B1 expression is not a pre-requisite to achieve a pCR with a neoadjuvant regimen including T-DM1. We plan to prospectively test cyclin B1 induction as a pharmacodynamics assay (i.e. serial biopsies comparing baseline cyclin B1 expression with expression at an early time point after T-DM1 treatment) to predict T-DM1 clinical benefit in a GEICAM (Spanish Breast Cancer Research Group) study in a near future.

A few additional mechanisms of T-DM1 resistance have been proposed. For instance, those factors that reduce the intracellular DM1 load per cell, namely the over-expression of multi-drug resistance (MDR) proteins or an impaired lysosomal degradation of trastuzumab that might limit the subsequent release of DM1 into the cytoplasm (3,35). Although we cannot rule out a potential role of these mechanisms in our models, we believe that MDR did not play a significant role since paclitaxel, a microtubule-stabilizing agents and a substrate of MDR, exhibited the same cytotoxicity in parental and in resistant cells.

With regards to alterations in the endosome pathways, we believe that the detection of intracellular T-DM1 in the resistant cells, as well as the effects of cdc20 silencing on restoring T-DM1 response in resistant cells, limits the potential role of this putative mechanism of resistance. Nonetheless, a biparatopic HER2-targeting ADC containing the
tubulysin variant AZ13599185 demonstrated superior anti-tumor activity over T-DM1 in various tumor models including T-DM1 refractory. The new ADC by targeting two non-overlapping epitopes on HER2 can induce HER2 receptor clustering, which in turn promotes robust internalization, lysosomal trafficking, and degradation (36). Also, it has been suggested a combination of inhibitors of the chaperone HSP90 that promote HER2 targeting to lysosomes and its degradation suggested as a new strategy to improve therapy with T-DM1 (37). In addition to this, other novel anti-HER2 antibody drug conjugate (ADC) offer promising activity in T-DM1 pretreated breast cancer (38,39). Other potential mechanisms of resistance that come from the trastuzumab part of the T-DM1 molecule have not proven its clinical utility when assayed in patient samples from clinical trials (9,11). A mechanism of T-DM1 resistance that has been reported pre-clinically is the presence of the HER3 ligand, heregulin, that reduced the activity of T-DM1 in breast cancer cells by causing HER2/HER3 dimerization thus strongly activating the PI3K pathway; and this effect was reversed by the addition of pertuzumab, a HER2-HER3 dimerization inhibitor. However, the combination of T-DM1 and pertuzumab in the clinic has not proven sufficiently superior to T-DM1 alone, suggesting this mechanism operates at most in a small proportion of patients (40). Finally, T-DM1 is able to alter genes related with immune response and this can influence the response in patients (41).

In short, the studies presented here show that cyclin B1 induction allowed to differentiate T-DM1 sensitive parental cells from their counterparts with acquired resistance. The induction of cyclin B1 in T-DM1 sensitive cells was independent of their prior trastuzumab sensitivity. The mitotic arrest consequence of the failure in assembling the mitotic spindle leads to sustained CDK1/Cyclin B1 kinase activity, a hallmark of mitotic catastrophe that is resolved by apoptosis. In fresh human HER2 positive breast cancer explants, the induction of cyclin B1 by T-DM1 correlates with apoptosis, suggesting that a pharmacodynamic assay to test cyclin B1 induction in breast cancer patients treated with T-DM1 may help to identify early the patients more likely to benefit from this drug.
Acknowledgments

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FIGURE LEGENDS

Figure 1. Generation and growth characteristics of HER2-positive breast cancer cells with T-DM1 acquired resistance.

(A) Pulsed treatment strategy for the *in vitro* establishment of acquired resistant cells to T-DM1. The procedure consisted of three consecutive cycles of 3 days on treatment followed by 3 days off treatment for each T-DM1 concentration of 1, 2 and 4µg/mL. The entire 54-day protocol resulted in the generation of cells with varying levels of T-DM1 resistance. (B) Growth characteristics of T-DM1 resistant cells. Each resistant cell line was compared with the corresponding age and passage-matched parental cell line. HCC1954-HCC1954/TDR, HCC1419-HCC1419/TDR, and SKBR3-SKBR3/TDR pairs of sensitive and resistant cells were seeded in duplicate in 12-well-plates at a density of 8,000 to 12,000 cells per well and allowed to adhere overnight. Then, cells were treated without (white columns) or with two concentrations of T-DM1 (black columns) for 3 days. Cells were collected by trypsinization and counted using a Scepter cell counter (Millipore). Experiments were performed in triplicate and data represent the mean ± SD cell number relative to control. Error bars denote standard deviation. (C) T-DM1 resistant cells retain a similar sensitivity to trastuzumab. Each paired sensitive/resistant cell lines were seeded as in panel (B) and treated in duplicate with or without 15 μg/mL trastuzumab for 7 days. Medium changes were performed every 3 days. Cells were trypsinized, collected, and counted using Scepter. The results are presented as mean % of viability in treated vs non-treated controls ± standard deviation.

Figure 2. Pharmacodynamics characterization of T-DM1-resistant HER2 positive breast cancer cells.

(A) Fluorescence *in situ* hybridization (FISH) assay for HER2 gene amplification. Paraffin-embedded cell pellets were prepared from each resistant cell line (HCC1419/TDR, SKBR3/TDR and HCC1954/TDR; right panel) and the corresponding age and passage-matched parental cells (HCC1419, SKBR3 and HCC1954; left panel). The level of HER2 amplification in cell nuclei was determined as the ratio HER2/CEP17. Nuclei are
highlighted by the blue fluorescent counter stain (4', 6-diamidino-2-phenylindole [DAPI]). Nuclei with high levels of HER2 gene amplification (red signal) are shown. Green signal (CEP17): centromere of chromosome 17. (Scale bar: 10 μm). (B) HER2 protein expression in parental and T-DM1 resistant cells. Expression of HER2 in whole-cell lysates from the cell pairs under basal growth. β-actin served as internal loading control. Western blot is from a representative experiment. (C) Flow cytometric analysis of T-DM1-receptor binding in parental and T-DM1 resistant cells. Cell line pairs were incubated with 7.5 μg/mL Rituximab (a humanized anti-CD20 IgG1 monoclonal antibody, as a negative isotype control) or 7.5 μg/mL of T-DM1, for 30 minutes on ice. Trastuzumab at 7.5 μg/mL was used as positive control. Phycoerythrin (PE)-conjugated Goat F(ab′)2 Anti-Human immunoglobulin was used as a secondary reagent. Representative flow cytometry histograms are shown. (D) T-DM1 internalization analysis. Cells were incubated with 1.5 μg/mL T-DM1 at 37°C for 15 minutes. In some conditions, cells were co-treated with chloroquine (50 μM) to accumulate intracellularly T-DM1. Cells were fixed and stained with Cy3-conjugated anti-human (for T-DM1 detection; red), FITC-phalloidin (for distribution of the F actin filaments; green) and DAPI (a nuclear DNA stain; blue). (Scale bar: 7.5 μm).

Figure 3. Cellular effects of T-DM1 in parental and resistant cells.

(A) The action of T-DM1 on cell cycle distribution. Cell cycle status was analyzed using BrdU incorporation and propidium iodide (PI) to assess DNA content by flow cytometry. Sensitive (HCC1954 HCC1419 and SKBR3) and T-DM1 resistant (HCC1954/TDR, HCC1419/TDR and SKBR3/TDR) cells were seeded into 6-well plates at a density of 2 × 10^5 cells/well for 24 hours. Cells were then treated without (Control) or with T-DM1 [0.1 μg/mL] for 24 hours and pulsed for an hour with BrdU prior to cell harvest and analysis. In the left panel, shown are representative flow cytometry plots from an experiment performed in triplicate that is consistent with other biological replicates. In the right panel, the bar chart shows the percentage of cells in the Go/G1, S, and G2/M phases of the cell cycle following T-DM1 treatment. A bar chart with statistics for each pair...
of cell lines showing the percentage of change in G2/M population after T-DM1 treatment assigning a value of 100% to the untreated condition is shown. (*P < 0.05, **P < 0.01, ***P < 0.001). **(B) Effects of T-DM1 in microtubules.** The three pairs of sensitive and T-DM1 resistant cells were grown at low density on cover slips, and incubated with T-DM1 [0.1 µg/mL] for 24 hours. Cells were washed, fixed and permeabilized before incubation with anti-α tubulin FITC conjugated antibody. The nuclei were counterstained with DAPI. Representative confocal images of cells visualizing the organization of tubulin filaments (green) are shown. The right panel is a magnification of the picture shown and a phase contrast image of the same area. (Scale bar: 24µm). **(C). T-DM1 is able to induce apoptosis in responsive cells.** The three pairs of sensitive and resistant cells were treated with or without T-DM1 [0.1 µg/mL] for 48 hours. The cells were harvested, washed with PBS and subsequently stained with annexin-V conjugated to FITC. Viable [annexin-V-/PI-], pre-apoptotic [annexin-V+/PI-], apoptotic [annexin-V+/PI+], and the residual damaged [annexin-V-/PI+] cells were quantified by flow cytometric analysis using the Muse Cell Analyzer. Representative flow cytometry dot plots of three independent experiments are shown. Annexin V in combination with PI can discriminate between among early apoptotic cells, late apoptotic cells, and cells that are in either the very late stages of apoptosis or necrosis. Graphs show the average percentage of indicated subsets of cells from three experiments. A bar chart with the average percentage of total cell death from three experiments with statistics is shown. (*P < 0.05, ***P < 0.001).

Figure 4. Effects of T-DM1 on CDK1/cyclin B1 kinase activity and expression in different HER2 positive breast cancer preclinical models.

**(A) T-DM1 is able to up-regulate the expression of cyclin B1 in a number of T-DM1 sensitive HER2-positive breast cancer cell lines.** Cells were treated with T-DM1 [0.1 µg/mL] for 24 hours. Protein expression levels of cyclin B1 and CDK1 was evaluated by western blot using 25 µg of protein cell lysate. The anti-β-actin antibody was used to verify equal protein loading. Representative image of three separate experiments is shown.

**(B). Effects of T-DM1 on CDK1/cyclin B1 kinase activity and expression in HER2-positive...**
breast cancer cell with acquired resistance to trastuzumab. Acquired trastuzumab-resistant cells (BT474/TR, SKBR3/TR, AU565/TR and EFM-192A/TR) have been generated by our group and used in these experiments together with the corresponding age and passage-matched parental cell line (20). Cells were treated with T-DM1 [0.1 µg/mL] and harvested at 24 hours. Whole-cell extracts were prepared from each experimental condition and CDK1/cyclin B1 kinase activity was measured with the non-radioactive MESACUP® Cdc2/CDK1 Kinase Assay. The results are reported as fold-induction of OD 492 nm in arbitrary units (RLA) of treated sample using untreated condition as reference set at 1. Data are presented as mean ± SD for three experiments. Cells treated as in the CDK1/cyclin B1 kinase activity assay experiment were lysed and western blots were performed with equal amounts of cell lysate (25 µg protein). Expression of cyclin B1 and CDK1 was evaluated. β-actin was used as an internal control. Shown are representative images from one of experiments. (*P < 0.05, **P < 0.01). (C). Trastuzumab is not able to up-regulate the expression of cyclin B1 in a number of T-DM1 sensitive HER2-positive breast cancer cell lines. Cells were treated with trastuzumab [15 µg/mL] and T-DM1 [0.1 µg/mL] for 24 hours and analyzed as in (A). Shown are representative images from one of experiments. (D). Effects of T-DM1 on CDK1/cyclin B1 kinase activity and expression in pairs of HER2-positive sensitive and T-DM1 resistant breast cancer cells. HCC1954 and HCC1954/TDR, HCC1419-HCC1419/TDR and SKBR3-SKBR3/TDR cells were treated with T-DM1 [0.1 µg/mL] for 24 hours and analyzed for CDK1 kinase activity using the MESACUP® Cdc2/CDK1 Kinase Assay Kit and cyclin B1 levels by western blot as in panel 3C. Data are expressed as fold induction versus control arbitrarily set at 1. (*P < 0.05, ***P < 0.001).

Figure 5. Genetic modulation of cyclin B1 affects the viability of cancer cells treated with T-DM1.

(A) Depletion of cyclin B1 in the parental drug-sensitive cells reduced T-DM1 sensitivity. Parental T-DM1 sensitive cells were transfected with target specific cyclin B1 siRNA and scrambled control siRNA by using a Nucleofector. After transfection, cells were recovered and used for experiments. Viability experiments were performed on an aliquot of
transfected cells after seeding in 12-well plates at a concentration of 10-15 × 10^3 cells/well and allowed to adhere overnight. Cells were then incubated with T-DM1 [0.1 µg/mL]. Duplicated samples were harvested and counted at 48 hours with the Scepter automated cell counter. The results are presented as the mean ± SD from experiments replicated five times (***P< 0.01, **P< 0.001). The remaining transfected cells were analyzed by western blot. Total cellular protein was extracted from cells at 48 hours after siRNA transfection. Western blots confirmed that cyclin B1 was down regulated. β-actin was blotted for loading control. (B) Increase of cyclin B1 in the drug-resistant cells augments their response to T-DM1. T-DM1 resistant cells were transfected with target specific cdc20 siRNA and scrambled control siRNA by using a Nucleofector. After transfection, cells were recovered and used for cell viability and Western blot experiments. Cells were incubated with T-DM1 [0.1 µg/mL]. Cell number was determined after 72 hours post-transfection with Scepter. The results are presented as the mean ± SD from triplicate experiments (*P< 0.05, ***P< 0.001). Lysates were prepared 48 and 72 hours post-transfection and analyzed for the levels of cyclin B1 and cdc20 by western blot.

Figure 6. Effects of T-DM1 added ex vivo to fresh human breast cancer explants. Formalin-fixed paraffin-embedded (FFPE) blocks were prepared for control and treated breast tumor at five days after the T-DM1 treatment.

(A). Graphs showing the percentage of cells positive for each marker. Cyclin B1, Caspase 3-active, Ki67 and p-H3 were determined in basal control condition and after treatment with T-DM1 for 5 days. (B) Representative immunohistochemistry images of control and T-DM1 treated tumors. Two representative examples of staining for cyclin B1 and caspase 3-active (apoptosis) are shown. (Scale bar: 50 µm). These tumors were also treated with trastuzumab [15 µg/mL] and the results are also shown. (C) Hierarchical cluster analysis of T-DM1 effects on cyclin B1 and Caspase-3 active in a total of 18 human breast cancers. Each row represents an effect on expression of the paired control- and T-DM1-treated samples, and each column, a single immunohistochemical marker, including cyclin
B1 and caspase 3-active. Up-regulation of expression is displayed in red, and no effect in white.
References


Figure 1

A

T-DM1 [µg/mL] 18 days

Parental cells

HCC1954

T-DM1 [2µg/mL] 18 days

HCC1954/TDR

T-DM1 [4µg/mL] 18 days

T-DM1 acquired resistant cells

B

Cell number (% of Control)

T-DM1 [µg/mL]

HCC1954

T-DM1 [2µg/mL]

HCC1954/TDR

T-DM1 [4µg/mL]

HCC1954/TDR

HCC1419

HCC1419/TDR

SKBR3

SKBR3/TDR

C

Cell number (% of Control)

HCC1954

HCC1954/TDR

HCC1419

HCC1419/TDR

SKBR3

SKBR3/TDR

Control

Trastuzumab
**Figure 2**

A. Parental vs Resistant

B. HER2 and β-actin expression in parental and resistant cells.

C. Flow cytometry analysis of % of Max for HCC1954, HCC1419, and SKBR3.

D. Immunofluorescence images of T-DM1 and Chloroquine-24h effects in different cell lines.
Figure 4

A

B

C

D
Figure 5

A

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**siControl**

**siCyclin B1**

**β-actin**

Cyclin B1

Cell number (% of Control)

T-DM1 [µg/mL]

0 0.1

0 0.1

0 0.1

B

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**siControl**

**siCdc20**

**β-actin**

Cdc20

Cyclin B1

Cell number (% of Control)

T-DM1 [µg/mL]

0 0.1

0 0.1

0 0.1

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Figure 6

A

Cyclin B1  c-casp3  Ki-67  p-H3

Baseline  T-DM1  Baseline  T-DM1  Baseline  T-DM1  Baseline  T-DM1

B

Control  Trastuzumab  T-DM1

C

Cyclin B1  c-casp3  KI-67

Annotation

4  16  1  17  18
6  2
7  5  8  10  12  9  14  3  13  11  19

-5  0  +5

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Defective cyclin B1 induction in trastuzumab-emtansine (T-DM1) acquired resistance in HER2-positive breast cancer

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