Title: mTOR inhibition and trastuzumab-emtansine (T-DM1) in HER2-positive breast cancer

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Running title: T-DM1 and mTOR inhibitors in HER2-positive breast cancer

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ABSTRACT

In patients with trastuzumab-resistant HER2-positive breast cancer, the combination of everolimus (mTORC1 inhibitor) with trastuzumab failed to show a clinically significant benefit. However, the combination of mTOR inhibition and the antibody-drug conjugate (ADC) trastuzumab-emtansine (T-DM1) remains unexplored. We tested T-DM1 plus everolimus in a broad panel of HER2-positive breast cancer cell lines. The combination was superior to T-DM1 alone in four cell lines (HCC1954, SKBR3, EFM192A, and MDA-MB-36) and in two cultures from primary tumor cells derived from HER2-positive patient-derived xenografts (PDX), but not in BT474 cells. In the trastuzumab-resistant HCC1954 cell line, we characterized the effects of the combination using TAK-228 (mTORC1 and 2 inhibitor) and knockdown of the different mTOR complex components. T-DM1 did not affect mTOR downstream signaling nor induct autophagy. Importantly, mTOR inhibition increased intracellular T-DM1 levels, leading to increased lysosomal accumulation of the compound. The increased efficacy of mTOR inhibition plus T-DM1 was abrogated by lysosome inhibitors (chloroquine and bafilomycin A1). Our experiments suggest that BT474 are less sensitive to T-DM1 due to lack of optimal lysosomal processing and intrinsic resistance to the DM1 moiety. Finally, we performed several *in vivo* experiments that corroborated the superior activity of T-DM1 and everolimus in HCC1954 and PDX-derived mouse models. In summary, everolimus in combination with T-DM1 showed strong antitumor effects in HER2-positive breast cancer, both *in vitro* and *in vivo*. This effect might be related, at least partially, to mTOR-dependent lysosomal processing of T-DM1, a finding that might apply to other ADCs that require lysosomal processing.

Implications: Inhibition of mTOR increases the anti-tumor activity of T-DM1, supporting that the combination of mTOR inhibitors and antibody-drug conjugates warrants clinical evaluation in patients with HER2-positive breast cancer.

KEY WORDS

mTOR inhibitors, everolimus, Trastuzumab-emtansine (T-DM1), Trastuzumab resistance, HER2-positive breast cancer

INTRODUCTION

HER2 gene amplification or protein overexpression (HER2 positivity) occurs in approximately 15-20% of breast cancers and confers poor prognosis (1). However, the addition of anti-HER2 antibodies to standard chemotherapy significantly improved the outcome of HER2-positive breast cancer patients (1–3). More recently, antibody-drug conjugates (ADC), combining the specificity of monoclonal antibodies with the cytotoxic potential of chemotherapeutic drugs, have proven highly effective in breast cancer (4,5). Ado-trastuzumab emtansine, also called T-DM1, consists of a potent anti-tubulin maytansinoid (DM1) bound by a non-cleavable linker to the monoclonal antibody trastuzumab. T-DM1 was the first approved ADC for therapy in solid tumors and is a standard of care for patients with metastatic HER2-positive breast cancer that have progressed to first line trastuzumab-based therapy or have residual disease following neoadjuvant anti-HER2 based therapy (6,7). Still, a considerable proportion of patients may present with *de novo* or acquired resistance to anti-HER2 agents. Consequently, the pursuit of novel strategies that increase response rates and/or delay the appearance of therapy resistance remains one of the primary interests of research in this field.

In an elegant preclinical study, mTOR inhibition greatly improved the antitumor effects of trastuzumab (8). The mammalian target of rapamycin (mTOR) protein is a main downstream effector of PI3K signaling. mTOR signals through two different multiprotein complexes termed mTORC1 and mTORC2 (9). Two ensuing randomized clinical trials testing the combination of trastuzumab-based therapy plus everolimus (a mTORC1 inhibitor) in HER2-positive breast cancer patients lead to statistically significant improvements in progression-free survival, but considering the modest magnitude of the benefit and the added side effects, it did not reach routine clinical practice (10,11). The combination of mTOR inhibitors and T-DM1 remains unexplored, likely in part due to the results mentioned above.

In contrast to trastuzumab, T-DM1 requires receptor-mediated internalization and entrance into the endolysosomal pathway. Once T-DM1 reaches the lysosome, it suffers proteolytic degradation by lysosomal proteinases leading to the release of lysine-linked DM1. Linker-bound DM1 then traverses the lysosomal membrane and accesses the cytosol to exert its antimitotic effect through binding to tubulin (12). Consistent with this, altered T-DM1 intracellular trafficking and lysosomal processing has been postulated as one of the main resistance mechanisms to T-DM1 (13–17). Other mechanisms of resistance that have been described are decreased HER2 expression, upregulation of multidrug transporters, loss of Cyclin B1 induction and increased PI3K pathway activation (17,18).

Due to the important role that lysosomal processing plays in the mechanism of action of T-DM1 and since mTOR is a master regulator of lysosome dynamics (9,19,20), we felt that the combination of T-DM1 with mTOR inhibitors deserved testing. Here, we examined the effects of mTOR inhibition plus T-DM1 in a wide panel of HER2-positive breast cancer cells, including two patient derived xenograft (PDX) primary culture models. The combination was superior to T-DM1 or everolimus alone in HCC1954, EFM192A, MDA-MB-361 lines, and in two cultures from primary tumor cells derived from HER2-positive patient-derived xenografts (PDX), but not in BT474 and SKBR3 cells. In BT474 and in the trastuzumab-resistant HCC1954 cell line, we characterized the biochemical and cellular effects of mTOR inhibition as well as lysosomal dynamics in different experimental conditions. Finally, we confirmed the superior antitumor activity of T-DM1 plus everolimus *in vivo* in subcutaneous and orthotopic HCC1954 mouse xenografts and in engrafted cells from PDX-derived primary cultures.

MATERIALS AND METHODS

Cell lines and Reagents

Breast cancer cell lines HCC1954, SKBR3, BT474, EFM192A and MDA-MB-361 were obtained from the ATCC. Authenticity of the cells was tested by STR DNA profiling analysis at the ATCC. PDX118 and PDX433 were obtained from de Vall d'Hebron Institute of Oncology. These PDX models were established from sites of distant metastases in Vall d'Hebron Institut of Oncology (21,22) and recently used by our group. PDX-118 was established after the patient received systemic treatment with Trastuzumab, Lapatinib and standard chemotherapeutics and it is from a skin biopsy. PDX-433 was a hepatic metastasis where the patient received Trastuzumab and T-DM1. The HCC1954 and EFM192A were grown in RPMI and SKBR3 and BT474, PDX118 and PDX433 were grown in DMEM: F12, supplemented with 10% fetal bovine serum (FBS), and containing high glucose (4500 mg/liter) and antibiotics (penicillin 100 U/ml, streptomycin 100 µg/ml). MDA-MB-361 were grown in DMEM supplemented with 20% FBS and antibiotics (penicillin 100 U/ml, streptomycin 100 µg/ml). Cell lines were cultured at 37°C in a humidified atmosphere in the presence of 5% CO_2 and 95% air. The number of passages between thawing and use in the described experiments was fifteen or less. Detection of mycoplasma was conducted at cell culture core facility of our institution. T-DM1 (trastuzumab-emtansine, Kadcyla) was provided by the Hospital del Mar pharmacy (Barcelona, Spain). Everolimus, TAK-228, bafilomycin A1 and chloroquine phosphate were obtained from Selleckchem. DM1 (mertansine) (HY-19792/CS-5804) was obtained from MedChemExpress. For *in vitro* studies, drugs were prepared at 10mM in DMSO and stored at -20ºC. Chloroquine phosphate was prepared

at 100mM in water and stored at -20ºC. T-DM1 was prepared at 20mg/ml in water and stored at -20ºC. For *in vivo* studies, everolimus was prepared in PEG400 and T-DM1 was prepared in physiological serum. IgG1 isotype control from Sigma-Aldrich (I5154) was prepared in physiological serum.

Cell viability assays

Cells were plated in triplicate in 12-well plates at a density of 15,000 to 70,000 per well (based on the optimal density for each cell line) in 1ml of culture medium. After 24 hours, the cells were treated with drugs, either alone or in combination, for 72 hours. Two different approaches (automated cell counting or crystal violet staining) were used to assess the effects of different treatments on cell viability. The cells were trypsinized and resuspended in PBS for counting using the Scepter Automated Cell Counter (Millipore, Billerica, MA). For the experiments with crystal violet, at the end of the experiments, cells were stained with crystal violet solution (10% acetic acid, 10% absolute ethanol and 0.06% crystal violet) for 1 hour. After that, the plates were washed with PBS. Images of each plate were scanned and quantified using ImageJ and plotted as arbitrary units.

Western blotting

Western blots were performed according to standard protocols. Cells were plated at a density of 8x105 to 1,2x106 in 100 mm2 dishes (based on the optimal density for each cell line) and, after 24 hours, cells were treated with drugs as indicated in figure legends. The following antibodies were used: p-Akt XP (Ser473) (4060), Akt (9272), p-S6 (Ser235/236) (2211), S6 (2217), LC3B-I-II (2775), p62/SQSTM1 (5114), cleaved-PARP (9541), p-ULK1 (Ser757) (6888), ULK1 (8054), LAMP1 (9091), raptor and mTOR from Cell Signaling Technology, cathepsin B (sc-6493) from Santa Cruz, α-tubulin (T5168) and β-actin (A-5316) (Sigma-Aldrich), rictor (Bethyl laboratories) and calnexin (Stressgen Biotechnologies Corporation). Anti-mouse (NA931) and anti-rabbit (NA934) horseradish peroxidase (HRP)-conjugated secondary antibodies purchased from GE Healthcare Life Sciences.

T-DM1 was analyzed by immunoprecipitation with protein A-Sepharose, followed by western blotting with antibodies to either human Ig (that recognize trastuzumab) or anti-DM1 antibodies. The latter were prepared in-house by injecting rabbits with keyhole limpet hemocyanin-coupled DM1, followed by purification of the specific anti-DM1 antibodies by affinity chromatography over a column prepared with BSA-copled DM1.

shRNA transfections

Lentiviral infection was performed as described in (23). The lentiviral vectors containing short hairpin RNA (shRNA) for raptor, rictor, and mTOR were obtained from Addgene.

Cell cycle and apoptosis analysis

Cells were plated at a density of $8x10^5$ in 100 mm^2 dishes and after 24 hours cells were treated with the different drugs. For cell cycle analysis, cells were fixed by 70% of ethanol O/N and then stained with the Muse™ Cell Cycle reagent (Merck Millipore) during 30 minutes at RT in the dark. Stained cells were analyzed by Muse Cell Analyzer (Millipore, Hayward, CA, USA) according to manufacturer's protocol. Apoptosis was assayed by determining cleaved Poly-ADP-Ribose (cleaved-PARP) by western blot.

Immunofluorescence and phase microscopy

Lysosome number was measured by Lysotracker Red DND-99 (Molecular Probes) staining according to manufacturer's protocol. Internalization of T-DM1 and its colocalization with lysosomes (i.e. intracellular accumulation) were measured by detecting T-DM1 and LAMP1 as described in (13). Briefly, acidic organelle staining was followed with 100 nmol/L LysoTracker Red DND-99, which was added 30 minutes before fixing. Phase contrast images were obtained using conventional photomicroscopy.

***In vivo* experiment**

All animal work was conducted following the PRBB Institutional Animal Care and Scientific Committee guidelines. Five-weeks-old female BALB/c nude mice were subcutaneously inoculated in their flank or in the mammary fat pad with $1.5x10^5$ (subcutaneous) and $1.5x10^6$ (orthotopic) HCC1954 cells mixed with matrigel. Five-weeks-old female NOD/SCID mice were subcutaneously inoculated in their flank with $5x10^6$ PDX118 cells mixed with matrigel. Tumor growth was measured two or three times a week depending on its growth. When tumors reached approximately 100-200 mm^3 mice were randomized to four groups. Treatment groups are indicated in figure legends. T-DM1 was administered at 1 mg/kg by intravenous injection on days 1, 22 and 43 of treatment. Everolimus was administered at 1 mg/kg by oral gavage five times a week. At the end of the experiment, animals were sacrifized and tumor tissue was harvested frozen or formalin-fixed and paraffin-embedded (FFPE).

Immunohistochemistry

FFPE fixed samples blocks were cut in 3µm tissue sections were immunostained in a Dako Link platform (pH3 and cleaved-caspase 3) or HER2 IHC assays PATHWAY® (Ventana/Roche) (HER2). The following antibodies were used: cleaved-caspase 3 (9664) and pH3 (9701) from cell signaling and HER2 (790-2991) from Roche. The percentages of cleaved-caspase 3 and pH3 positive tumor cells for each case were determined according to our own procedures (24). Xenograft Tumor HER2 expression was determined by Ventana's HER2 IHC assays PATHWAY, an FDA-approved in vitro diagnostic test marketed by Roche.

Quantitative real time polymerase chain reaction (qRT-PCR)

Human primer pair sequences are *SLC46A3* Forward: 5'-TTGGATTCACCACTCTGCTG-3' and Reverse: 5'-GAGGCACTACCCAAAGCTGA-3' (25); *GAPDH* Forward: 5'-GGAGTCAACGGATTTGGTCGTA-3' and Reverse: 5' GGCAACAATATCCACTTTACCAGAG-3' . Gene expression was calculated as 2 to the power of - ΔΔCt, where $\Delta\Delta Ct = (Ct_{Gene} - Ct_{ATP5E \text{ or } GAPDH})$ Assay.

Statistics and plotting

Statistical analysis was performed by SPSS version 18.0 (SPSS, Inc.). For *in vitro* experiments, differences in means between conditions was assessed using one-to-one t-tests, with the null hypothesis being no differences between groups. For *in vivo* experiments two-way ANOVA with post-hoc Tukey test for pairwise differences was performed. Contrasts with a p-value <0.05 were considered statistically significant. Significant group-group differences are depicted in corresponding plots using different thresholds, as described in the figure legends. Barplots and Boxplots were designed using Graph Pad Prism® v6.05. The boxplot in Figure 4F was generated using the ggplot2 library on R Software version 4.3.0.

RESULTS

Effects of T-DM1 plus everolimus on HER2-positive breast cancer cell viability

Everolimus forms a complex with FKBP12, binding to the FRB (FKBP12-Rapamycin-Binding) domain of the mTORC1 complex and acting as a selective allosteric inhibitor of mTOR (9). We tested the effects of T-DM1 with and without everolimus on the cell viability of seven HER2-positive breast cancer cell lines with different therapeutic profiles: two trastuzumab-resistant cell lines (HCC1954 and MDA-MB-361), three trastuzumab-sensitive lines (SKBR3, EFM192A, and BT474), and in two cultures from primary tumor cells derived from HER2-positive patient-derived xenografts (PDX), PDX118 and PDX433 (21,22) (see Methods and Supplementary Figure 1A). We used everolimus at 100 nM because at this concentration its effect on cell viability plateaued (Supplementary Figure 1B) (26). In all cells but BT474, addition of Everolimus to T-DM1 caused a significant decrease in cell viability compared with T-DM1 alone (Figure 1A and Supplementary Figure 1C). In SKBR3 cells, the activity of the combination was not significantly superior compared with everolimus alone.

Since T-DM1 strongly depends on lysosomal processing to exert its cytotoxic effect (12), we reasoned that the addition of chloroquine (CHQ) would decrease T-DM1 efficacy. CHQ is a dibasic lipophilic amine drug that passively diffuses through cell membranes. When CHQ reaches the acidic lumen of lysosomes, it becomes protonated and consequently trapped (27,28), impairing lysosomal function. Of note, CHQ by itself has shown to inhibit cell proliferation and induce apoptosis in preclinical breast cancer models at concentrations of 10μM or higher (29,30). Therefore, we chose a relatively low concentration of CHQ (5 μM) to avoid any significant impact on cell viability while still impacting lysosomal function. Overall, we observed a decrease in T-DM1 anti-tumor effects after the addition of CHQ (Figure 1A). This was observed in all cell lines except MDA-MB-361, in which notably T-DM1 alone also caused no significant decrease in cell numbers. Furthermore, CHQ did not impact cell viability or modify the activity of everolimus, coherent with the antiproliferative effect of everolimus not depending on lysosome function. In HCC1954, EFM192A and MDA-MB-361 cells, CHQ also reverted the effects of the T-DM1 plus everolimus (TE) combination, although this effect was modest. Findings from these initial experiments were compatible with our initial hypothesis, namely that everolimus increases T-DM1 efficacy in HER2-positive breast cancer. However, our observations in BT474 suggested that certain cells may be resistant to the TE combination.

We subsequently decided to study the TE combination in a trastuzumab-resistant model. Based on the literature, HCC1954 cells are trastuzumab-resistant *in vitro* (31) and *in vivo* (32). Thus, we henceforth focused on this model to further characterize the effects of the TE combination. Furthermore, our findings in BT474 cells suggested that our results may not be universal across all HER2-positive breast cancer models. Hence, we ran parallel experiments in BT474 cells to explore potential mechanisms of resistance to the combination. We present these data in a dedicated section in the text, in Figure 4 and Supplementary Figure 3.

Role of lysosomal inhibitors and mTOR complex on T-DM1 efficacy in HCC1954 cells

To confirm the role of lysosome inhibition in T-DM1 anti-tumor activity, we first replaced CHQ with the lysosomal proton pump V-ATPase inhibitor bafilomycin A1 (Supplementary Figure 1D). We observed a significant abrogation of both T-DM1 and TE activity, supporting the notion that impaired lysosomal function decreases T-DM1 efficacy (13,14). Second, we substituted T-DM1 for unconjugated DM1 (uDM1), which freely penetrates cell membranes and thus does not need lysosomal processing to inhibit tubulin polymerization. Like TE, the combination of uDM1 and everolimus significantly decreased cell viability compared to each drug alone (Figure 1B). In contrast with the experiments with T-DM1, the addition CHQ did not revert the effect of uDM1 alone or in combination with everolimus.

Next, to confirm the involvement of mTOR as a regulator of the antitumoral efficacy of T-DM1, we repeated the previous experiments replacing everolimus with TAK-228, an ATP-competitive mTOR kinase inhibitor (33). As shown in Figure 1C, treatment with TAK-228 mimicked the results of the experiments with everolimus in terms of cell viability. Next, we explored T-DM1 efficacy after knockdown of different components of the mTOR. We either silenced Raptor (Regulatory protein associated with mTOR) or Rictor (rapamycin insensitive companion of mTOR), which are essential components of mTOR complex 1 and 2, respectively. We also knocked down the mTOR kinase, which abrogates signaling through both complexes. Western Blot confirmed a significant decrease of each knocked down component (20.7% mTOR kinase vs. control, 80.2% Raptor vs. control and 49.8% Rictor vs. control) which was preserved in conditions with T-DM1 (34% mTOR kinase vs. control, 74% Raptor vs. control and 40.4% Rictor vs. control) (Figure 1D, left). Notably, we observed a significant decrease in cell viability by knocking down any of the mTOR components in the absence of T-DM1 (Figure 1D, right), which was consistent with our previous results with everolimus and TAK-228. In this

regard, the largest effect was observed after knockdown of the mTOR kinase. Addition of T-DM1 further decreased cell viability in all conditions. However, this decrease after the addition of T-DM1 was only significant after either mTOR kinase or Raptor knockdown. Together, the results from both pharmacologic and genetic inhibition of mTOR suggested that the effects of the TE combination were primarily due to mTORC1 inhibition.

T-DM1 internalization and lysosomal accumulation with and without everolimus

To study the role of everolimus in T-DM1 intracellular trafficking, we first analyzed T-DM1 intracellular accumulation, a trait that was associated with sensitivity to T-DM1 in a recent study (13). Indeed, we observed an increased accumulation of T-DM1 with the addition of everolimus (Figure 2A). We then analyzed the influence of each treatment on lysosomal biogenesis by using LAMP1 (a specific marker of lysosomal membrane) and cathepsin B (a lysosomal enzyme) levels as surrogate markers of lysosome number. As expected, the addition of everolimus increased LAMP1 and Cathepsin B levels, an effect that was sustained over time (Figure 2B). Furthermore, everolimus also increased lysosome number as measured by LysoTracker Red immunofluorescent staining, which occurred independently of T-DM1 (Figure 2C). We did not observe significant changes in lysosome numbers with T-DM1 alone.

Next, we studied T-DM1 internalization and its colocalization with lysosomes. Cells were treated with a 15-minute pulse of T-DM1, after which the drug was washed out (Figure 2D). Immediately after the pulse, most T-DM1 was bound to the cell membrane. After 24h, most T-DM1 had been processed or externalized and consequently, T-DM1 staining decreased. With the addition of everolimus, intracellular T-DM1 concentration increased and was mainly localized to the lysosomal compartment. Interestingly, we observed a clearly higher amount of intracellular T-DM1 in the conditions with CHQ. We speculate that this is due to the lysosomal blockage caused by CHQ on the final steps of T-DM1 processing, and that what we observe corresponds to the high numbers of T-DM1 molecules that appear sequestered in the lysosomal compartment. Furthermore, this amount appears to be greater in the condition with everolimus, suggesting that everolimus may increase cellular uptake of T-DM1. This would be consistent with previous studies showing increased ligand-receptor internalization after mTOR inhibition (34). Together with the results from the previous experiments, these results suggested that mTOR inhibition increases intracellular T-DM1 levels, leading to increased lysosomal processing of the drug.

Effects of T-DM1 plus everolimus on mTOR downstream signaling

To study the integrity of the mTOR pathway in the different experimental conditions, we analyzed the phosphorylation of S6 ribosomal protein ($pS6_{S235/236}$) and Akt ($pAkt_{Ser473}$), which are downstream effectors of mTORC1 and mTORC2, respectively. Consistent with its mechanism of action, everolimus abrogated phosphorylation of S6 (Figure 3A). The effect of everolimus on the pathway was not altered by T-DM1, uDM1, CHQ, or bafilomycin A1 (Figure 3A and Supplementary Figures 2A and 2B) TAK-228 also decreased the levels of pS6, although to a lesser extent than everolimus (Figure 3B) and this was not significantly altered in conditions T-DM1 or CHQ.

In contrast to everolimus, TAK-228 also decreased phosphorylation of AKT, which is consistent with its dual mTORC1 and mTORC2 inhibition. Therefore, the effects observed on the mTOR pathway were coherent with the known mechanisms of action of everolimus and TAK-228. These effects did not differ appreciably with the addition of any of the other compounds, including T-DM1.

Effects of T-DM1 plus everolimus on cell cycle, apoptosis, and autophagy

We then studied the effect of the TE combination on apoptosis induction and cell cycle modifications. According to the literature and our previous experience (18), T-DM1 causes mitotic arrest at G2/M phase and ultimately cell death due to mitotic catastrophe. We did not observe a significant increase in G2/M cells with the TE combination with respect to the other conditions. Rather, a predominant increase of cells in G0/G1 was observed in the presence of everolimus, which persisted in the TE condition (Figure 3C). These results did not differ significantly when experiments were repeated at 48h (Supplementary Figure 2C). Regarding apoptosis, we observed similar levels of cl-PARP between T-DM1 alone and the TE combination (Figure 3D). Thus, the cumulative decrease in cell viability observed with T-DM1 after mTOR inhibition in HCC1954 does not appear to be caused by a clear increase in apoptotic or G2/M cells.

Besides regulating lysosomal biogenesis, mTOR also regulates autophagy (9,20). Autophagy is an evolutionarily conserved catabolic process that mediates the degradation of large protein aggregates or damaged organelles (35). In recent years, autophagy has gained momentum as a crucial mechanism for cancer cell survival and therapeutic resistance (36). Inhibition of mTOR is a *bona fide* method to induce autophagy. In this context, lysosome inhibitors such as CHQ or bafilomycin A1 are commonly used to assess variations in autophagic flux, since they block the final stages of the autophagic process (37). To assess if the effect of the TE combination was dependent on autophagic activity, we assessed autophagic flux in the different

experimental conditions and using different markers. Briefly, we analyzed LC3B-I/LC3B-II turnover as well as changes in SQSTM1/p62 and pULK1 levels (37). An increase of the LC3B-II/LC3B-I (lipidated/unlipidated LC3B) ratio is used to measure autophagosome formation and is especially apparent in the presence of late-phase autophagy inhibitors such as CHQ. Indeed, we observed a clear increase in LC3B-II accumulation in the presence of everolimus and TAK-228, which was not evident with T-DM1 alone (Figure 3E and F, Supplementary Figure 2D and 2E). Degradation of p62 is another widely used marker to monitor autophagic activity because p62 directly binds to LC3B and is degraded during the autophagic process, and Atg1/pULK is involved predominantly in the induction of autophagy by mTOR. Coherent with the changes observed in LC3B-II, we observed a decrease of p62 and pULK in the conditions with everolimus and TAK-228. CHQ (5 μM) caused the expected increase in LCB3I/II ratio and the expected changes in p62, namely a slight accumulation or stability (similar to control or T-DM1 conditions) when compared to conditions in which autophagy is inducted (everolimus and TAK-228) (Supplementary Figure 2D and E). Importantly, T-DM1 alone (with or without CHQ) did not appear to induce or inhibit autophagy. These findings suggest that autophagy activation was preserved in all our experimental conditions and was primarily driven by mTOR inhibition.

Effects of T-DM1 plus everolimus in BT474 cells

Despite not observing a significant decrease in cell viability with the addition of mTOR inhibitors to T-DM1 in BT474 cells (Figures 1A and 4A), results thus far showed that the effects of T-DM1 with and without mTOR inhibition regarding mTOR downstream signaling (Figure 4B), autophagy (Supplementary Figure 3A) as well as T-DM1 internalization and accumulation (Figures 4C, D and Supplementary Figure 3B) were overall consistent with those observed in HCC1954 cells.

Furthermore, and in contrast to what we observed with the TE combination, uDM1 plus everolimus did show a cumulative decrease in cell viability in BT474 cells (Figure 4E). However, consistently with our previous experience (18) and that of other groups (13,15), we had to employ a 10-fold higher T-DM1 concentration in BT474 than in HCC1954 to achieve a similar decrease in cell viability. Similarly, higher uDM1 concentrations were needed in BT474 to achieve effects similar to the ones observed in HCC1954. Together, these findings suggest that BT474 may be less sensitive to T-DM1 both by lacking optimal lysosomal processing of T-DM1 as well as by having intrinsic resistance to the DM1 moiety. With regard to the former, several studies have highlighted the potential role of specific lysosomal proteins such as V-ATPase or SLC46A3 (16,17,38) in T-DM1 resistance. Such reports suggest that SLC46A3 expression is required for exporting the

product of cleaved T-DM1, lysine-MCC-DM1, from the lysosome to the cytosol (16,17), a role that appears to be specific to all non-cleavable linker-based ADCs (16,38). Interestingly, we explored the expression of SLC46A3 in our cell lines and found that BT474 expression of the transporter is among the lowest within HER2-positive cells, while HCC1954 has one of the highest (Figures 4F and G).

Activity of T-DM1 and everolimus on trastuzumab-resistant tumor xenografts

We established three tumor xenograft models to test the *in vivo* antitumor activity of the TE combination. Cells were injected subcutaneously in the flank (HCC1954 and PDX118), as well as orthotopically in the mammary fat pads (HCC1954) of immune deficient mice. Mice were then treated with either T-DM1, everolimus alone or the combination of both drugs (TE). Everolimus was administered at 1 mg/kg by oral gavage five times per week, based on published data (39). As shown in Figures 5A-C, TE was superior to T-DM1 in all models. In the PDX118 model, this difference did not reach a statistical significance. Of note, engrafted tumors from these cells grew much slower than those from HCC1954. We also noted that PDX118 cells were more sensitive to everolimus than HCC1954, which we had also confirmed *in vitro* (Supplementary Figure 1B).

Tumors treated with TE showed a decrease in mitotic markers and increased apoptosis, as determined by phospho-histone H3 and cleaved Caspase 3 levels, respectively. In addition, the tumors treated with the combination showed ischemic necrosis. The results of the subcutaneous xenografts are shown in Figure 5D and the results of the orthotopic xenografts are shown in Supplementary Fig 4A. Similar findings were observed in the PDX118 xenografts (Supplementary Fig 4B). Lastly, it has been debated that HER2 downregulation plays an important role in the efficacy of anti-HER2 treatments (17,40–42). In HCC1954 models, we found no effect on HER2 expression neither *in vitro* (Supplementay Figure 4C) nor *in vivo* (Supplementary Figure 4D) in any of the experimental conditions. Altogether, the results from the *in vivo* models corroborated the increased efficacy of TE compared with T-DM1 alone, by decreasing cell division and increasing tumor cell apoptosis.

DISCUSSION

Here we report that T-DM1 plus everolimus in HER2-positive breast cancer is superior to T-DM1 alone in a wide range of HER2-positive cell lines. Interestingly, we observed significant activity of the combination with a relatively low concentration of T-DM1, compared with our previous experience with T-DM1 in the same cell lines (18). We observed in 5 out of 7 cell lines that the combination of T-DM1 and everolimus had a

greater effect on cell viability that either T-DM1 or everolimus alone. Our results suggest that the improved effect of TE may be a common effect in HER2-positive breast cancer, but not universal. We selected HCC1954 cells for further studies because i) they were sensitive to the combination, ii) they are trastuzumab-resistant, and iii) they grow well *in vivo*. Indeed, we found promising anti-tumor activity of T-DM1 plus everolimus in both subcutaneous and orthotopic HCC1954 mouse models. We also validated the results of the TE combination in an *in vivo* model of PDX-derived primary culture cells stemming from distant metastases from a patient treated with trastuzumab and lapatinib. Together, our findings indicate that TE warrants further investigation in the clinical setting.

Most clinical experience with mTOR inhibition in HER2-positive breast cancer comes from studies in combination with trastuzumab, since activation of the PI3K/mTOR pathway was shown to drive resistance to trastuzumab in preclinical models (8). Two studies addressed this question by adding everolimus to trastuzumab and chemotherapy, failing to show a significant benefit compared to trastuzumab alone (BOLERO-1) (10), or reporting a modest clinical benefit at the expense of increased toxicity (BOLERO-3) (11). The results of these studies may explain the relative scarcity of clinical trials combining mTOR inhibitors and T-DM1. In contrast, promising evidence regarding the combination of T-DM1 with PI3K inhibitors is emerging. At the time of writing this manuscript, at least two clinical trials of T-DM1 with PI3K inhibitors had been published. In this context, the PI3Kα isoform-specific inhibitor alpelisib was tested in a phase I trial, with six out of 14 patients (43%) showing an objective response. Of note, three of the responding patients (out of 10 included in the study) had received previous T-DM1 (43). Similarly, a response was observed in eight out of 24 patients participating in a phase I trial with taselisib and T-DM1, and they seemed to occur independently of previous T-DM1 treatment and/or *PIK3CA* mutation (44). Other trials testing the combination of T-DM1 and PI3K inhibitors are ongoing (NCT02390427, NCT00928330). Interestingly, the design of these studies appears to be based on the same hypothesis as the BOLERO studies, namely that PI3K activation is a common resistance mechanism to early-line anti-HER2 therapy. To the best of our knowledge, there is only one study, reported thus far only as abstract (45), that has previously reported increased activity of T-DM1 with mTOR inhibition in a trastuzumab-resistant breast cancer model. Importantly, however, it was conducted on only two cell lines (KPL4 and MCF7 neo/HER2) different from any of those employed in our study, and the experimental drug (GDC-0980) was a dual PI3K/mTOR inhibitor.

The different effects observed in each cell line used in our study may be explained by their different molecular backgrounds. Subgroup analyses of the BOLERO-1 and BOLERO-3 trials revealed that only patients with hormone-receptor negative disease derived a significant benefit from the addition of everolimus (10,11,46). Similarly, a molecular analysis of patients included in both studies showed that only patients whose tumors harbored a 'hyperactive' PI3K pathway (defined as the presence of *PTEN* loss or PI3KCA activating mutation in tumor tissue) benefitted from everolimus plus trastuzumab-chemotherapy (47). Notably, the benefit with everolimus in HER2-negative breast cancer appears to be independent of PI3K pathway status (48), suggesting that this phenomenon is restricted to HER2-positive disease. Of the cell lines employed in our study, HCC1954 and SKBR3 are hormone-receptor negative, while BT474, EFM192A and MDA-MB-361 are hormone-receptor positive. All but SKBR3 (*PTEN* loss) harbor a pathogenic *PIK3CA* mutation, although only HCC1954 and MDA-MB-361 occur in *bona fide* gain-of-function hotspots (p.H1047R and p.E545K, respectively). Interestingly, the effect of adding everolimus was highest in these two cell lines. In contrast, we found that the combination failed to increase the cell viability effects compared to T-DM1 or everolimus alone in BT474 and SKBR3 cells. As they differ in hormone receptor status, we cannot suggest that this drives resistance to the combination. In our experiments. BT474 cells appeared to be less sensitive to T-DM1 both by lacking optimal lysosomal processing as well as by having intrinsic resistance to the DM1 moiety. Interestingly, we found that BT474 express significantly lower SLC46A3 than HCC1954, which has been reported to regulate the transport of insoluble linker-bound DM1 from the lysosome to the cytosol (16,38). Importantly, no mutation has been reported in this gene in any of the cell lines used in our study. Lastly, we also observed differences in sensitivity to everolimus between HCC1954 and PDX118 cells, both *in vitro* and *in vivo*. Notably, the subcutaneous HCC1954 model was less sensitive to everolimus alone than the orthotopic one. Of note, we observed that HER2 remained highly expressed in HCC1954 models, both *in vitro* and *in vivo*. Altogether, our findings highlight that disease biology, beyond hormone receptor status, must be taken into consideration to select patients for therapy with T-DM1 and mTOR inhibitors.

Two recent preclinical studies, one conduced in HER2-positive breast- and another in HER2-positive gastric cancer, found that T-DM1 strongly inhibits mTOR and induces autophagy (30,49). Interestingly, they reported discordant results in anti-tumor activity with T-DM1 and the early-stage autophagy inhibitor LY294002. Zhang et al. found an increased anti-tumor activity of T-DM1 plus LY294002 vs. T-DM1 alone in subcutaneous NCI-N87 gastric cancer xenografts, concluding that LY294002 abrogates cytoprotective

autophagy caused by T-DM1 (49). In contrast, Liu et al. reported that LY294002 reverts T-DM1 anti-tumor activity in BT474 and SKBR3 cells *in vitro*, hypothesizing that the autophagy induced by T-DM1 facilitates apoptosis (30). Findings from these two studies stand in contrast with our results since we did not find any significant effect of T-DM1 on mTOR downstream signaling or autophagy, despite observing consistent antitumor activity across several models. Of note, both studies assessed the effects of T-DM1 on mTOR and autophagy using T-DM1 concentrations >10-fold higher than the ones we employed in our experiments. Indeed, no alterations of mTOR downstream signaling or in LC3B-II/I ratio were observed by Liu et al. when they used T-DM1 concentrations similar to ours (30). Taking these findings into account, we postulate that i) the effects of T-DM1 on mTOR signaling and autophagy may be concentration-dependent, and ii) biological differences between HER2-positive gastric- and breast cancer may dictate the different fate of cells treated with T-DM1 and autophagy inhibitors. Of note, and despite CHQ being a well-known autophagy inhibitor, we used it in our experiments to highlight role of lysosomal processing in T-DM1 activity. Importantly, together with the data reported by Liu et al. (30), our results do not support the use of T-DM1 in combination with autophagy inhibitors in HER2-positive breast cancer.

To summarize, our results support that the combination of T-DM1 with mTOR inhibitors is active in multiple HER2-positive breast cancer models. This effect might be related, at least partially, to mTOR-dependent lysosomal processing of T-DM1. Our findings might be applicable to other ADCs such as trastuzumab deruxtecan, which has recently proven highly effective in patients with HER2-positive breast- (5) and gastric cancer (50). In fact, the superiority of trastuzumab deruxtecan compared to T-DM1 will shift the clinical use of T-DM1 to later lines in HER2-positive advanced breast cancer. Hence, T-DM1 and mTOR inhibitors combinations might be of importance in this new clinical setting.

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MAIN FIGURE LEGENDS

Figure 1. Effects of T-DM1 in combination with mTOR inhibition on cell viability. A. Sensitivity of breast cancer cells to T-DM1 in combination with everolimus in presence or absence of chloroquine. Cells were treated with T-DM1 [HCC1954, SKBR3, EFM192A, MDA-MB-361: 0.01µg/ml; BT474: 0.1µg/ml], everolimus [100nM], or with the combination of both drugs in presence or absence of chloroquine phosphate (CHQ) [5µM]. Cell viability was measured using the automatic Scepter 2.0 counter at 72 hours. The number of viable cells in each treatment was plotted as a percentage of the control. **B-C. Combination of maytansinoid and mTOR inhibition in HCC1954 cells.** Cells were treated with everolimus [100nM], uDM1 [2nM] and CHQ [5 µM] **(B)**, T-DM1 [0.01µg/ml], TAK-228 [25nM] and CHQ [5 µM] **(C)** alone or in combination. After 72 hours, cells were fixed and stained with 0.06% crystal violet. Images of each plate were scanned and quantified. Blue color intensity was measured in arbitrary units using ImageJ. The number of viable cells in each treatment was plotted as a percentage of the control. **D**. **Sensitivity of HCC1954 cells to T-DM1 in mTOR knockdown conditions.** HCC1954 cells were transfected with the indicated shRNAs. Cells were treated with T-DM1 [0.01µg/ml]. Cell lysates were assayed using western blot with the indicated antibodies (left). The number of viable cells in each treatment was plotted as a percentage of the control (right). Significant differences between treatment conditions are depicted according to different p-value thresholds: * $p<0.5$, ** $p<0.01$ and *** $p<0.001$. For clarity purposes, differences between any of the conditions vs. their corresponding controls are not shown, even if they were present.

Figure 2: T-DM1 accumulation and lysosomal activity with everolimus. A. DM1 Detection after the treatments with T-DM1 and everolimus. Levels of DM1 were analyzed by western blot after immunoprecipitation using protein A-sepharose. Cells were treated with everolimus [100nM] and T-DM1 [0.01µg/ml]. **B. Effects of everolimus on lysosomal proteins.** HCC1954 cells were treated with everolimus [100nM] for 24, 48 and 72 hours. Cell lysates were assayed using western blot with LAMP1 and cathepsin B. Relative density of the bands was normalized to α-tubulin. Representative images of two separate experiments are shown. The graphs show the levels of LAMP1 and cathepsin B in each condition expressed as fold induction versus each control arbitrarily set at 1. **C. Lysosome quantification.** Lysosome number was measured by Lysotracker Red DND-99 staining after the treatment with the drugs, everolimus [100nM] and T-DM1 [0.01µg/ml]. The graph shows the mean fluorescence intensity in each condition. **D. Internalization of T-DM1 and its colocalization with lysosomes.** Cells were pulsed with T-DM1 [0.01µg/ml] for 15 minutes at 37ºC, chased for 24 hours. And costained with Anti-Human-Dylight 488 (T-DM1; Green) and the acidic vesicle indicator Lysotracker red. Scale bar: 25 µm. Colocalization between T-DM1 and lysotracker is shown in white (fourth row). Image-generated scatter plots of acquired images for colocalization analysis were processed by the LAS AF software (last row). Pure red and green pixels are between abscissa/ordinate and white lines; colocalizating pixels are found inside central region of the plot (between white lines). LysoTracker/T-DM1 colocalization, calculated as the ratio of the area of colocalizing signals with respect to total fluorescence area, is indicated.

Figure 3. Molecular and cellular effects of T-DM1 plus mTOR inhibitors. HCC1954 cells were seeded in 100mm2 dishes and 24 hours later, cells were treated with everolimus [100nM], T-DM1 [0.01µg/ml], TAK-228 [25nM] or chloroquine phosphate CHQ [5µM] alone or in combination for 24 hours. **A and B. Molecular effects of the drugs on the PI3K/AKT/mTOR signaling pathway.** Cell lysates were assayed using western blot with the indicated antibodies. Signals were quantified with the ImageJ and normalized to α-tubulin or β-actin. Densitometry values are the ratio between phosphorylated and total protein in each condition, expressed as fold induction versus control (±CHQ) arbitrarily set at 1. Representative images from three different experiments are shown. **C. Effects of T-DM1 combined with everolimus on cell cycle distribution.** Fixed and stained cells were analyzed by Muse Cell Analyzer. The bar chart shows the percentage of cells at different cell stages. **D. Effects of T-DM1 in combination with everolimus on apoptosis.** HCC1954 cells were treated with the drugs for 48 hours. Cleaved-PARP antibody was used as a marker of apoptotic cells. Signals were quantified with the ImageJ and normalized to α-tubulin. The graphs show the cleaved-PARP expression in each condition, expressed as fold induction versus control arbitrarily set at 1. **E and F. Effects of the drugs on the autophagy pathway.** Cell lysates were assayed using western blot with LC3B. Signals were quantified with the ImageJ and normalized to α-tubulin or β-actin. Graphs show the densitometry values of the ratio between LC3B-II and LC3B-I in each condition, expressed as fold induction versus control (±CHQ) arbitrarily set at 1.

Figure 4. Molecular and cellular effects of T-DM1 in combination with mTOR inhibition in BT474 cells. Cells were treated with T-DM1 [0.1µg/ml], TAK-228 [25nM], everolimus [100nM], uDM1 [8nM] and CHQ [5µM] as indicated. **A. Sensitivity of BT474 cells to T-DM1 in combination with TAK-228.** Cells were treated with T-DM1 and TAK-228, and the combination of the drugs in presence or absence of CHQ. Cell viability was measured using the automatic cell counter at 72 hours. **B. Molecular effects of the drugs on the PI3K/AKT/mTOR signaling pathway.** Cell lysates were assayed using western blot with the indicated antibodies. Signals were quantified with the ImageJ and normalized to α-tubulin. Densitometry values are the ratio between phosphorylated and total protein in each condition, expressed as fold induction versus control (±CHQ) arbitrarily set at 1. **C. Effects of everolimus on lysosomal proteins.** Cells were treated with everolimus for 24, 48 and 72 hours. Cell lysates were assayed using western blot with LAMP1 and cathepsin B. Relative density of the bands was normalized to α-tubulin. Representative images of two separate experiments are shown. The graphs show the levels of LAMP1 and cathepsin B in each condition expressed as fold induction versus each control arbitrarily set at 1. **D. Intracellular accumulation of T-DM1 in lysosomes.** BT474 cells plated on coverslips were pulsed with T-DM1 for 15 minutes at 37°C and chased for 0 and 1 day. Cells were fixed and stained for T-DM1 (red), LAMP1 (green) and DNA (blue) and analyzed by confocal microscopy. Scale bar: 7.5 µm. **E. Sensitivity of BT474 cells to uDM1 in combination with everolimus.** Cells were treated with uDM1 and everolimus, and the combination of the drugs in presence or absence of CHQ. Cell viability was measured using the automatic cell counter at 72 hours. **F. SLC46A3 expression levels across HER2-positive breast cancer cell lines.** The y-axis shows RNAseq Log2 transformed RSEM expression counts (using a pseudo-count of 1) of *SLC46A3* across HER2-positive cell lines. Cell lines used in our study are depicted in

red. Data was downloaded from the Broad Institute Cancer Cell Line Encyclopedia at the DepMap web portal (https://depmap.org/portal/download- accessed January 14, 2022). **G. Basal mRNA expression of SLC46A3 in HCC1954 and BT474.** Analysis of SLC46A3 gene expression by qRT-PCR. Gene expression levels were normalized to GAPDH as the housekeeping gene. Cell data were normalized to N87 cells expression level (as a positive control) set at 1 (dotted line). **p<0.01 and ***p<0.001.

Figure 5. *In vivo* effects of T-DM1 in combination with everolimus. A and B. Effects of the drugs on tumor growth in HCC1954 xenografts. Cells were injected subcutaneously in the flank **(A)** or in the mammary fat pad **(B)** of the mice. Mice were divided into four groups (five **(A)** or seven **(B)** animals in each group): control; T-DM1 1mg/Kg (days 1, 22 and 43 of treatment), everolimus 1mg/Kg (five times per week) and the combination of both drugs. In the fourth group, the combination group, animals were treated with T-DM1 and 24 hours later with everolimus, 5 times/week. A plot of average tumor volume as a function of time in each treatment group is shown. **C. Effects of the drugs on tumor growth on PDX118 xenografts.** Cells were injected subcutaneously in the flank of the mice. Animals were divided into four groups (five animals in each group). Treatments were performed as indicated in A and B. A plot of average tumor volume as a function of time in each treatment group is shown (left). Graph representing the tumor volume at the end of the experiment (right). **D. Effects of T-DM1 in combination with everolimus on cell cycle and apoptosis in tumor samples.** Representative IHC images of tumor samples stained for p-H3 and c-caspase 3 (left). Box plots illustrating the staining results (right). The graph expresses the percentage of p-H3 or c-caspase 3 positive cells. * p<0.05 and **p<0.01 Significant differences between treatment conditions are depicted according to different p-value thresholds: * p<0.5, **p<0.01 and ***p<0.001. For clarity purposes, differences between any of the conditions vs. their corresponding controls are not shown, even if they were present.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Effects of T-DM1 and everolimus alone or in combination on cell viability in HCC1954, PDX118 and PDX433 cells. Cells were treated with the indicated drugs and after 72 hours, cells were fixed and stained with 0.06% crystal violet. Images of each plate were scanned and quantified. Blue color intensity was measured in arbitrary units using ImageJ. The number of viable cells in each treatment was plotted as a percentage of the control. **A. Effects of T-DM1 on cell viability in PDX118 and PDX443.** Cells were treated with T-DM1 [0.01 and 0.1 µg/ml]. **B. Sensitivity of HCC1954 and PDX118 cells to everolimus.** Cells were treated with increasing doses of everolimus (0.01, 0.1, 1, 10, 100 and 1000). Values shown in the graph are the mean percentage +/- SD of cell viability relative to controls and plotted as logarithmic curves using GraphPad Prism Software. **C. Effects of the combination of T-DM1 with everolimus on cell viability in PDX118 and PDX433.** Cells were treated with T-DM1 [0.01µg/ml] and everolimus [100nM] or with the combination of both drugs. **D. Combination of T-DM1 and mTOR inhibition in HCC1954 cells.** Cells were treated

with T-DM1 [0.01µg/ml], everolimus [100nM] and bafilomycin A1 (Baf-1A) [1nM] alone or in combination. * p<0.05, **p<0.01 and ***p<0.001.

Supplementary Figure 2. Molecular and cellular effects of maytansinoids plus mTOR inhibitors in HCC1954 cell line. Cells were seeded in 100mm2 dishes and 24 hours later, cells were treated with the indicated drugs. **A-B. Molecular effects of maytansinoids plus everolimus.** Cells were treated with T-DM1 [0.01µg/ml] or uDM1 [2nM], everolimus [100nM] or with the combination of both drugs in presence or absence of CHQ [5µM] or bafilomycin A1 (Baf-1A) [1nM]. Cell lysates were assayed using western blot with the indicated antibodies. Signals were quantified with the *ImageJ* and normalized to α-tubulin or *β*-actin*.* Densitometry values are the ratio between phosphorylated and total protein in each condition, expressed as fold induction *versus* control (±CHQ or Baf-1A) arbitrarily set at 1. Representative images from three different experiments are shown. **C. Effects of T-DM1 combined with everolimus on cell cycle distribution.** HCC1954 cells were treated with T-DM1 [0.01µg/ml], everolimus [100nM] and the combination of the two drugs for 48 hours. Fixed and stained cells were analysed by Muse Cell Analyzer. The bar chart shows the percentage of cells at different cell stages. **D-E. Molecular effects of T-DM1 plus mTOR inhibitors.** HCC1954 cells were treated with T-DM1 [0.01µg/ml], everolimus [100nM] or TAK-228 [25nM], and with the combination of both drugs in presence or absence of CHQ [5µM]. Cell lysates were assayed using western blot with the indicated antibodies. Signals were quantified with the *ImageJ* and normalized to α-tubulin *or β*-actin*.* Densitometry values are expressed as fold induction versus control (±CHQ) arbitrarily set at 1. Representative images from three different experiments are shown.

Supplementary Figure 3. Molecular and cellular effects of T-DM1 in combination with mTOR inhibition in BT474 cells. A. Molecular effects of T-DM1 plus everolimus. BT474 cells were treated with T-DM1 [0.1µg/ml], everolimus [100nM], and the combination of both drugs in presence or absence of CHQ [5µM] for 24 hours. Cell lysates were assayed using western blot with the indicated antibodies. Signals were quantified with the *ImageJ* and normalized to α-tubulin Densitometry values are expressed as fold induction versus control (±CHQ) arbitrarily set at 1. Representative images from three different experiments are shown. **B. Lysosome detection.** Lysosome number was measured by Lysotracker Red DND-99 staining after the treatment with the drugs, everolimus [100nM] and T-DM1 [0.1µg/ml]. The graph shows the mean fluorescence intensity in each condition.

Supplementary Figure 4. *In vivo* effects of T-DM1 in combination with everolimus. A and B. Effects of T-DM1 in combination with everolimus on cell cycle and apoptosis in tumor samples. Representative IHC images of tumor samples stained for p-H3 and c-caspase 3. **A.** Tumor samples of HCC1954 xenografts. **B.** Tumor samples from PDX118 xenografts. **C. Molecular effects of T-DM1 plus everolimus on HER2 expression.** HCC1954 cells were seeded in 100mm^2 dishes and 24 hours later, cells were treated with everolimus [100nM], T-DM1 [0.01µg/ml] or with the combination for 24 hours. Cell lysates were assayed using western blot with the HER2 antibody ((AM134) from Biogenex). Signals were quantified with the ImageJ and normalized to α-tubulin. Densitometry values are expressed as fold induction versus control arbitrarily set at 1. Representative images from six different experiments are shown. **D. Molecular effects of T-DM1 plus everolimus on HER2 expression in tumor samples from HCC1954 xenografts.** Representative IHC images of tumor samples stained for HER2.