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Original Article

A tipping-point for apoptosis following dual inhibition of HER2 signaling network by T-DM1 plus GDC-0980 maximizes anti-tumor efficacy

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Abstract: HER2 signaling network and its complex relationship with the PI3K-AKT-mTOR pathway explain the acquired resistance to anti-HER2 therapy observed in clinics. Such complexity has been clinically evident from the limited efficacy of data in the BOLERO-1 and BOLERO-3 trials, which tested combinations of trastuzumab (T), everolimus, and chemotherapy in women with HER2+ advanced BC. In the following MARIANNE trial also, a combination of T-DM1 plus pertuzumab delivered a non-inferior but yet not superior PFS compared to trastuzumab plus a taxane. Algorithmic inhibition of PI3K/mTOR along with T or T-DM1 is, therefore, an attractive drug combination, and we tested the combination(s) in HER2+ BC, especially in T-resistant and *PIK3CA* mutated conditions. GDC-0980, a dual pan-PI3K/mTOR inhibitor alone or in combination with T or T-DM1, was examined in a panel of HER2+ T-sensitive (BT474, SKBR3), HER2+ T-resistant (BT474HerR), HER2+/*PIK3CA* mutant (HCC1954, MDA-MB453), and HER2+/*PTEN* mutant (HCC1569) BC cell lines. GDC-0980 re-sensitized trastuzumab-resistant, *PIK3CA* mutant, or *PTEN* mutant cells to T and acted additively with T. Importantly, this activity was more when GDC-0980 is combined with T-DM1. The combination (with T or with T-DM1) was then tested in the HER2+/T-sensitive, HER2+/T-resistant, and HER2+/*PIK3CA* mutated BC xenograft models for the anti-tumor effect. Along with its anti-tumor effect, GDC-0980 effectively decreased tumor angiogenesis (CD31 staining). Maximum anti-tumor (from tumor growth inhibition to tumor regression) efficiency was observed in all three xenograft models when T-DM1 was combined with GDC-0980. The anti-proliferative effects of GDC-0980 as evidenced by a decreased p-AKT (Ser473, The308), p-P70S6K, p-S6RP, and p-4EBP1, along with blockade of clonogenic 3D growth was accompanied by the initiation of apoptotic activity (annexin V, CASPASE3, cleaved PARP1 and mitochondrial depolarization); and was significantly superior when GDC-0980 combined with T-DM1. Interestingly, both trastuzumab and T-DM1 induce PD-L1 expression in *HER2* amplified BC cells. Our data provide evidence that an oncogenic mutation of *PIK3CA* and *HER2*-amplification may represent biomarkers to identify patients who may benefit most from the use of GDC-0980 and an opportunity to include immunotherapy in the combination of anti-HER2 therapy.

Keywords: Breast cancer, HER2+, *PIK3CA* mutation, angiogenesis, apoptosis, trastuzumab-sensitive, trastuzumab-resistant

Introduction

HER2 is the only validated marker to select breast cancer patients for anti-HER2 treatments; however, it is becoming evident that HER2+ breast cancer is a heterogeneous disease. The focus on HER2 expression/amplification status alone is not a realistic approach to understand the underlying mechanism of disease progression and resistance. More and more new HER2 treatments are becoming available. Amplification of *HER2* is recorded in approximately 20% of all invasive breast cancers in human beings and presents an increased risk of disease progression and poor overall survival [1]. HER2-positive breast cancers express higher expression of HER2 mRNA, as well as overexpression of the HER2 protein. The

HER2 transmembrane receptor transduces growth signals and acts as a driver of cell proliferation [2]. Amplification of HER2 increases ligand-independent receptor homodimerization as well as ligand-dependent heterodimerization (HER2-HER3) in the HER2 amplified tumor cells. The subsequent activation of downstream signaling pathways leads to cell proliferation, differentiation, survival, and angiogenesis [3]. It is now clear that although expression of HER3 is much lower than HER2 in HER2+ breast cancer, its expression is highly essential, and HER2 amplified tumors will not grow if HER3 is not present [4-6]. A heterodimer containing HER2 plus HER3 relies on HER3 for its signaling. The importance of HER3 may be at least partly related to its potent ability to activate the downstream PI3K-AKT-mTOR pathway [6-9]. In contrast, the HER2 homodimer lacks phosphorylated tyrosine in its cytoplasmic tail, preventing it from docking to the PI3K pathway adaptor protein p85 and activating PI3K signaling. Instead, engagement of the HER2 homodimer primarily activates the GRB2 and SHC adaptor proteins that initiate RAS/MAPK signaling [10], which is also known to activate p110α (the catalytic subunit of PI3K) [11].

Before the approval of trastuzumab [12] and lapatinib [13], HER2 amplification in breast cancer was associated with poor clinical outcomes [14-17]. More recently, additional anti-HER2 agents like pertuzumab [18] and trastuzumab emtansine (T-DM1) [19] have also been introduced into treatment regimens. Trastuzumab extends progression-free survival (PFS) and overall survival (OS) in both adjuvant and metastatic HER2 positive breast cancers [12, 20-22]. Several studies confirmed the reality that although HER2 targeted therapy (with chemotherapy) significantly improved outcomes, it did not cure HER2+ breast cancer. HER2+ breast cancer may be intrinsically or may become resistant to trastuzumab in around 70% of cases [23]. Indeed increasing knowledge regarding HER2 and its network has revealed multiple novel strategies to attack HER2 and its close network beyond trastuzumab. Recent literature suggests that alteration of the PI3K-AKT-mTOR pathway (activating mutation of *PIK3CA*) is a potential predictive marker of resistance to anti-HER2 treatment, even if a dual anti-HER2 treatment was given [24] and [25].

Antibody-drug conjugates are a class of drugs that use antibodies specifically targeting tumor-associated antigens as vehicles to deliver covalently attached small molecule toxins into cancer cells [26]. T-DM1 [27, 28] does not have typical adverse events of chemotherapy. Therefore, we have been interested in combining it with dual PI3K/mTOR inhibitor, GDC-0980 in HER2+/T-sensitive, and HER2+/T-resistant breast cancer models. Recently, DS-8201a (trastuzumab-deruxtecan), a novel ADC that connects trastuzumab via a tetrapeptide linkage to the topoisomerase-1 inhibitor, deruxtecan, received FDA accelerated approval in December 2019 for unresectable or metastatic HER2+ BC, who had received two or more prior anti-HER therapies.

Rapalogues (e.g., everolimus) were the first inhibitors of the PI3K pathway downstream effector (mTORC1) to enter the clinic [29]. The most impressive data on everolimus has been generated by a combination trial (BOLERO-2) with anti-endocrine therapy in estrogen receptor-positive breast cancer [30] and now routinely used in breast clinics. However, in the phase III BOLERO-1 trial reported by Hurvitz and colleagues that addition of everolimus (allosteric inhibitor of mTORC1) to trastuzumab/paclitaxel did not significantly increase progression-free survival (PFS) among patients with HER2+ metastatic breast cancer (in the full population, median PFS was 14.95 months in the everolimus group versus 14.49 months in the placebo group) [31]. Similarly, in the phase III BOLERO-3, which evaluated the addition of everolimus to trastuzumab and vinorelbine in the trastuzumab-resistant setting, showed a very modest improvement in PFS for just over 1 month (median PFS was 7.00 months with everolimus and 5.78 months with placebo) [32]. Preclinical studies demonstrated several negative regulatory mechanisms [33, 34] and other possible mechanisms of resistance triggered after blockade of mTORC1 by rapalogues, which potentially attenuate the activity of these therapeutic antagonists when used in the clinic. The limited anti-tumor activity of rapalogues (including everolimus) is known to be related to a well-documented mechanism of resistance due to the inactivation of the negative feedback loop on AKT, which may reactivate the PI3K pathway [33, 35] and rapalouges mediated mTOR inhibition is only restricted to mTORC1 but not

mTORC2. To bypass this feedback resistance, compounds able to inhibit both mTOR (mTORC1 and mTORC2) and its upstream PI3K were generated, including GDC-0980. This small molecule inhibits both PI3K and mTOR kinase activity by binding to the ATP binding cleft of these enzymes [36]. Their dual activity is based on the structural similarities of the catalytic domain of mTOR and the p110 subunit of PI3K, providing the potential advantage of targeting the pathway at two levels (blocking mTOR in both the mTORC1 and mTORC2 complexes and PI3K) [37]. We have previously shown that GDC-0980 can potentially inhibit cell proliferation and tumor growth inhibition in the triple-negative breast cancer model [38].

Here, we describe the preclinical anti-tumor efficacy of an orally available dual PI3K/mTOR inhibitor GDC-0980; as a single-agent or in combination with trastuzumab or T-DM1. The activity of the PI3K-AKT-mTOR pathway inhibitors in combination with classical anti-cancer agents has proved highly effective in several experimental systems [39-41]. Combining drugs targeting different nodal points of the same pathway is likely to be more effective in cells that are addicted to that pathway. We show GDC-0980 or, in combination with trastuzumab or T-DM1, inhibit PI3K-AKT-mTOR signaling, leading to inhibition of cancer cell viability in diagnostically defined HER2+ breast cancer cells characterized by trastuzumab-sensitive, trastuzumab-resistant, or PIK3CA activating mutation. Analysis of annexin V-positive cells, mitochondrial depolarization, and Western blot expression of cleaved CASPASE 3, cleaved PARP, and BIM indicated that GDC-0980-induced HER2+ breast cancer cell death through an apoptosis-dependent pathway. Furthermore, we show that GDC-0980 is efficacious as a single agent or in combination with trastuzumab or T-DM1 in all three xenografts (trastuzumab-sensitive, trastuzumab-resistant, or HER2+/*PIK3CA* mutated) models. Our studies provide a strong mechanistic rationale for the clinical development of GDC-0980 in the treatment of HER2+ breast cancer, particularly in trastuzumab-resistant breast cancer patients, along with trastuzumab or T-DM1.

Materials and methods

Cell culture

We have used human breast cancer cells (BT474, HCC1954, MDA-MB453, UACC893, and HCC1569). Cells were obtained from the American Type Culture Collection (ATCC). We also used BT474HerR, a trastuzumab-resistant breast cancer cell line. BT474HerR was kindly provided by Dr. Mark Pegram (Stanford University, CA). HER2 overexpressed BT474, and trastuzumab-resistant BT474HerR breast cancer cells were cultured in DMEM supplemented with 10% fetal bovine serum, 1% HEPES (Cellgro, Hemdon, VA) with 100 units/ml penicillin and streptomycin (Cellgro, Hemdon, VA) at 37°C in a humidified atmosphere containing 5% CO_2. HCC1954 and HCC1569 cells were cultured in RPMI1640 and Leibovitz's L-15 media (procured from ATCC), respectively, at 37°C in a humidified atmosphere containing 5% CO_2. Both the media were supplemented with 10% FBS with 100 units/ml penicillin and streptomycin. MDA-MB453 and UACC8963 cells were cultured in Leibovitz's L-15 media with 10% FBS and 100 units/ml penicillin and streptomycin. These cells were cultured in a non-CO_2 incubator.

Antibodies and reagents

We sincerely thank Roche-Genetech for providing GDC-0980, GDC-0941, trastuzumab, and T-DM1. Antibodies used include phospho-AKT (Ser473, Thr308), AKT, phospho-P70S6K, P70S6K, phospho-S6 ribosomal protein (S235/236), S6 ribosomal protein, phospho-4EBP1 (Thr37/46), 4EBP1, phospho-ERK (Thr202, Tyr204), cleaved CASPASE3 (Asp175), cleaved PARP1, and BIM were obtained from Cell Signaling Technology (Danvers, MA). A beta-ACTIN antibody was obtained from Sigma (St. Louis, MO). HRP-tagged anti-rabbit IgG, anti-mouse IgG, and Chemiluminescence Kit were from Amersham Pharmacia Biotech (Uppsala, Sweden). Recombinant human heregulin-β1 was purchased from Peprotech Inc. (Rocky Hill, NJ). Matrigel for tumor cell inoculation was purchased from BD laboratories. All other chemicals were purchased from Sigma (St. Louis, MO).

3D ON-TOP colony assay

We performed the 3D ON-TOP colony formation assay to examine the effect of GDC-0980 alone or in combination with trastuzumab or T-DM1 on the clonogenic growth of HER2+ breast cancer cells as initially described by Lee et al. [42]. Pictures of the live colonies were taken using an Olympus DP72 digital camera. Since HER2/

HER3 heterodimers are able to induce strong downstream signaling and activate various biological responses such as cellular proliferation and growth, and known as a HER3 ligand, heregulin (HRG)-β1-induced colony formation was also examined following GDC-0980 treatment in HER2+ breast cancer cells.

Biochemical analysis

Evaluation of the effect of dual PI3K/mTOR inhibitor GDC-0980 on the AKT-mTOR pathway activity: 10 cm^2 dishes were seeded with five million cells in a volume of 10 ml complete medium followed by incubation at 37°C under 5% CO_2 overnight (~16 hours). Cells were treated with the indicated concentration of GDC-0980 or GDC-0941 (pan-PI3K inhibitor) or RAD001 (mTOR allosteric inhibitor, from Selleclchem.com) for the time indicated. At the end of the treatment, cells were washed with cold PBS and solubilized with lysis buffer [50 mmol/L Tris-HCL (pH 7.6), 150 mmol/L NaCl, 100 mmol/L NaF, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.05% NP40, 1% aprotinin, 0.01 mg/mL leupeptin, and 0.08 mmol/L phenylmethylsulfonyl fluoride] for Western blots. For immunoblots, an equal amount of proteins were separated by SDS-PAGE, proteins were transferred into nitrocellulose membrane using the Criterion system and protocol from Bio-Rad (Hercules, CA).

Real-time confluency assay and apoptosis

A real-time proliferation of cells was documented using time-lapse phase-contrast imaging (IncuCyte; ESSEN BioScience). Proliferation was assessed using real-time microscopic images every 6 hours in an Essen IncyCyte Zoom. In brief, cells were plated at a low confluence on day one. Cells were treated with the indicated compounds on day two and imaged up to 100 hours of post-treatment. Using live cells, we generated long-term growth and growth inhibition curves and monitored morphology. The time course of the percentage of the confluence of non-treated and treated HER+ BC cells (mean vs. time) was represented by four days (time courses: 24 hours, 48 hours, 72 hours, and 96 hours) [43]. At the end of the treatment period, cells were trypsinized, and apoptosis was measured using BD AnnexinV-PE and 7aaD apoptosis kit. Cells were treated in triplicate with the indicated compounds and analyzed at 48 and 72 hours post-treatment. Early apoptotic (AnnexinV-PE positive, 7-aaD negative) events are represented graphically using Graph Pad Prism 6. In short, cells were resuspended in phosphate-buffered saline (PBS) containing 4 mM $CaCl_2$, annexin V-phycoerythrin (PE; BD Pharmingen, San Jose, CA), and 7AAD according to the manufacturer protocol. Cells were analyzed by flow cytometry (Accuri C6) [38]. For live/dead measurements in the IncuCyte, two reagents were used. AnnexinV (Essen BioScience) was added at the time of drug treatment and used to measure apoptotic cells over time. Vybrant Dye Cycle green was spiked in at specific time points as a live cell marker (Molecular Probes). Mitochondrial depolarization was determined as a biological readout of apoptosis following a combination of GDC-0980 and T-DM1. BT474 (A) and HCC1954 (B) cells were treated with indicated compounds for 48 and 72 hours. For mitochondrial membrane potential, staining of cells was done by treating them with 400 nM TMRE for 30 minutes at 37°C prior to trypsin release. Cells were rinsed and placed in FACS buffer (phenol red-free RPMI with 1% FBS) for collection and analysis by flow cytometry (BD Accuri C6). Staining controls included no TMRE and cells pretreated with 20 μM FCCP for 10 minutes prior to TMRE addition.

Animals for in vivo studies

Athymic female mice (*NCr*, 20-25 g) were used for all *in vivo* tumor growth inhibition studies. Mice (7-8 weeks old) were purchased from Harlan Laboratories and housed on a 12 h light/dark cycle with food and water *ad libitum* under specific pathogen-free conditions, following the guidelines of the Association for the Assessment and Accreditation for Laboratory Animal Care, International. Xenograft studies were approved by institutional IACUC, experimental animal care, and use protocols.

Tumor implantation

BT474, BT474HerR, and HCC1954 breast cancer cell lines were harvested from mid-log phase cultures using trypsin-EDTA (Invitrogen Inc.). Five million cells in 100 μl PBS/matrigel (1:1 mixture of DMEM and matrigel) were injected subcutaneously to generate tumor xenografts. Tumor growth was monitored twice per week for external measurements using Vernier

calipers. Tumor volume was calculated using the formula $V = (A \times B^2)/2$, where A and B represent the length and width of the tumor, respectively.

Treatment of mice with Trastuzumab, T-DM1, and GDC-0980

Treatment was initiated when tumors in all mice in each experiment ranged in size from ~200 mm^3 and were distributed into groups of no less than 8 animals/group, ensuring each group had equivalent mean tumor volumes before initialing dosing for anti-tumor efficacy studies. Control-1 mice received human myeloma IgG1 antibody (Calbiochem-Novabiochem, La Jolla, CA) 20 mg/kg twice-weekly by i.p. injection, and control-2 received 0.5% methyl cellulose/0.2% Tween-80 and GDC-0980 administered (10 mg/kg in 100 µl) through oral gavage. Trastuzumab (diluted in sterile PBS; 10 mg/kg) was administered twice per week. T-DM1 (10 mg/kg) was delivered by i.v. every 3 weeks. GDC-0980 was formulated at concentrations (10 mg/kg in 100 µl) in 0.5% methyl cellulose/0.2% Tween-80 to achieve the indicated dosages and was administrated every alternate day for 4 weeks with 100 µl via oral gavage.

Tumor volumes were determined by digital calipers using the formula (L × W × W)/2 and expressed as mean relative tumor volume (mm^3). Mice with tumor volumes >2000 mm^3 or with losses in body weight 20% or more from their weight at the start of treatment were killed per institutional IACUC guidelines. No untoward effects were noted in mice treated with trastuzumab, T-DM1, GDC-0980, a combination of trastuzumab plus GDC-0980, a combination of T-DM1 plus GDC-0980, or vehicles. All tumors were collected at the end of the experiments to maintain internal consistency.

Pharmacodynamic studies

The pharmacodynamic (PD) biomarkers were measured to ascertain whether GDC-0980 blocked PI3K-mTOR signaling in xenograft tumors. For the PD studies, tumors were analyzed for the markers shown at the end of the treatment period. We performed immunohistochemistry (IHC) on the paraffin-embedded tumor tissue sections. Expression of Ki67, CD31, phosphorylated vascular endothelial growth factor receptor (p-VEGFR), and phosphorylated-S6RP was determined on tumor tissues.

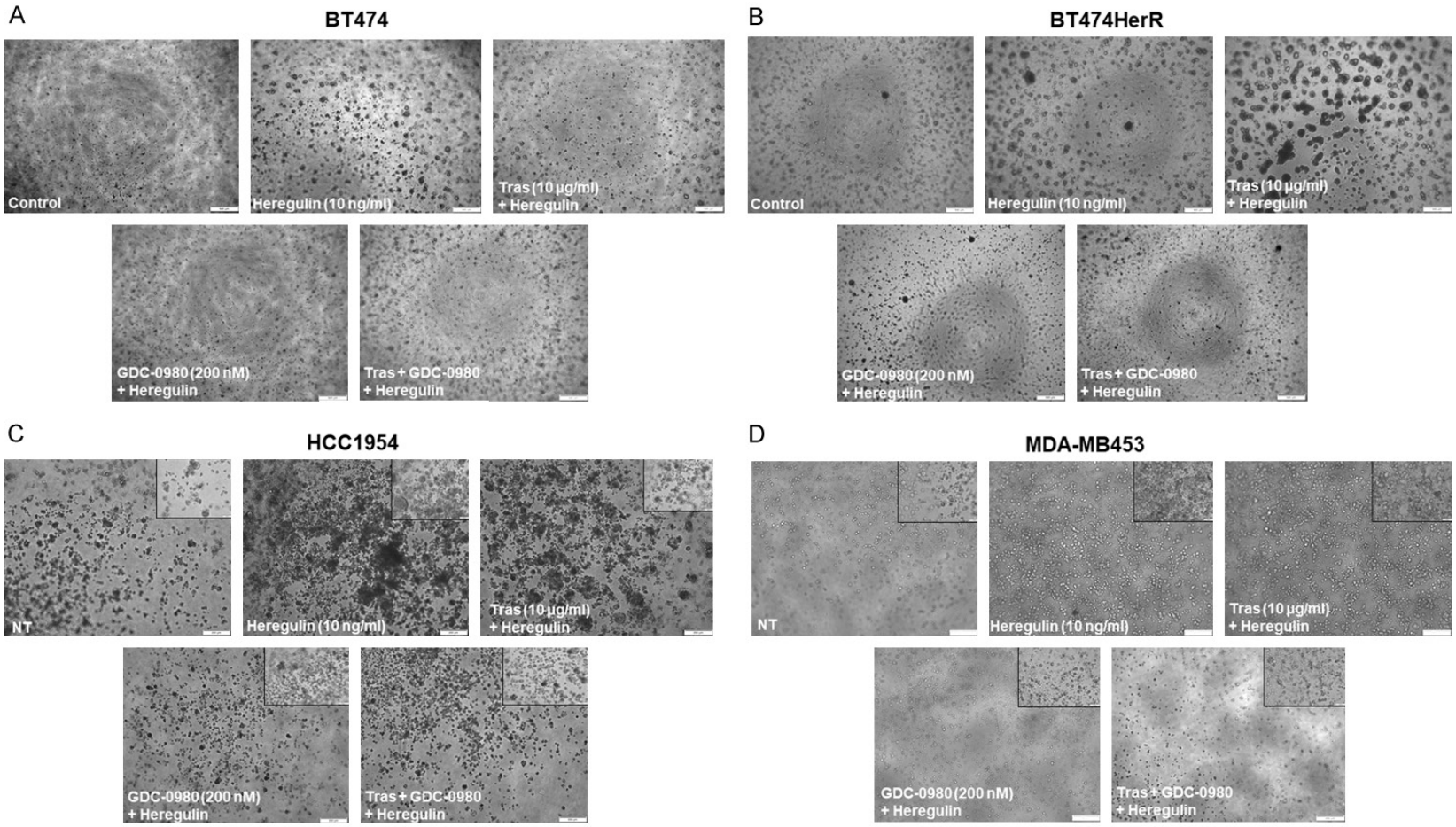
Statistical analysis

All *in vitro* experiments were performed at least three times independently in triplicates. Student's *t*-test is used to evaluate differences observed between treated groups and vehicle-treated controls. *In vivo* data are presented as means ± SE from eight mice/group and were analyzed by unpaired two-tailed Student's *t*-test. A *p*-value of $P<0.05$ was considered statistically significant.

Results

GDC-0980 inhibits proliferation of HER2 overexpressing breast cancer cells

We examined the effect of a combination treatment of GDC-0980 plus trastuzumab in a 3D-ON-TOP clonogenic assay in basal condition, or heregulin stimulated condition using BT474 (T-sensitive), BT474HerR (T-resistant), HCC1954, MDA-MB453, and MDA-MB361 (*PIK3CA* mutated) cells (**Figure 1A-E**). We repeated the experiment with HCC1954, where we used T-DM1 instead of trastuzumab (**Figure 1F**). Trastuzumab showed little to no inhibition of colony growth (in T-resistant and *PIK3CA* mutated cells), while GDC-0980 alone caused 50-70% growth inhibition (in heregulin stimulated conditions). Combined trastuzumab plus GDC-0980 achieved maximum inhibition (≥ 90%) of 3D colony growth than GDC-0980 alone in all cell lines (T-sensitive, T-resistant, and *PIK3CA* mutated) (**Figure 1A-E**). As expected, T-DM1 alone is highly effective in growth inhibition in *PIK3CA* mutated HCC1954 cells (**Figure 1F**). Thus, GDC-0980 appears to increase anti-HER2 therapy (either with trastuzumab or with T-DM1)-mediated growth inhibition in both basal and heregulin-induced conditions in HER2 overexpressing breast cancer cells, including T-resistant and *PIK3CA* mutated conditions. In **Figure 4Cii**, we showed that real-time proliferation was blocked in cells following T-DM1 plus GDC-0980 treatment in both BT474 (**Figure 4Ci**, left panel) and HCC1954 (**Figure 4Cii**, left panel), which led to the apoptosis at 48 hours (**Figure 4Ci**, **4Cii** right panels). As expected, more dead cells (red) were observed following the combination treatment of T-DM1 and GDC-0980 (**Figure 4Ci**, **4Cii**), bottom pan-


A
BT474
Control
Heregulin (10 ng/ml)
Tras (10 µg/ml) + Heregulin
GDC-0980 (200 nM) + Heregulin
Tras + GDC-0980 + Heregulin
B
BT474HerR
Control
Heregulin (10 ng/ml)
Tras (10 µg/ml) + Heregulin
GDC-0980 (200 nM) + Heregulin
Tras + GDC-0980 + Heregulin
C
HCC1954
NT
Heregulin (10 ng/ml)
Tras (10 µg/ml) + Heregulin
GDC-0980 (200 nM) + Heregulin
Tras + GDC-0980 + Heregulin
D
MDA-MB453
NT
Heregulin (10 ng/ml)
Tras (10 µg/ml) + Heregulin
GDC-0980 (200 nM) + Heregulin
Tras + GDC-0980 + Heregulin

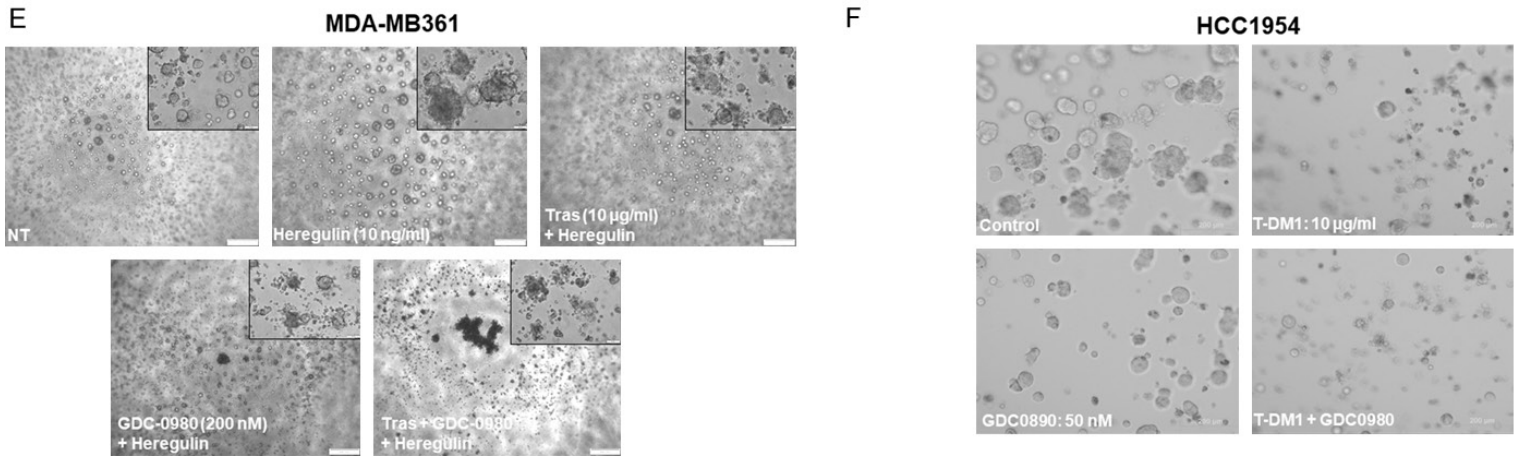


Figure 1. Effects of blocking of the PI3K-AKT-mTOR and HER2 signaling pathways in heregulin-induced 3D-ON-TOP clonogenic proliferation assays: Cells were treated with PI3K/mTOR dual inhibitor, GDC-0980 (200 nM), HER2 inhibitor trastuzumab (10 µg/ml)/T-DM (10 µg/ml) and a combination of GDC-0980 plus trastuzumab/T-DM1 for 7 days. Cells were then washed and stimulated with heregulin (10 ng/ml) for 24 hours. BT474, BT474HerR, HCC1954, MDA-MB453, and MDA-MB361 cells exhibit differential susceptibility to trastuzumab but are nearly equally sensitive to GDC-0980. Data clearly show that GDC-0980 is highly effective in all cell lines irrespective of *PIK3CA* mutation or not. Representative clonogenic survival of BT474 (A), BT474HerR (B), HCC1954 (C), MDA-MB453 (D), and MDA-MB361 (E) breast tumor cells was presented. The most potent inhibition of clonogenic growth was achieved when GDC-0980 plus T-DM1 combination was tested in HCC1954 cells (F).

els). We test the relationship between the effect of a combination of T-DM1 and GDC-0980 drug and apoptosis, and we tested the mitochondrial depolarization as a biological readout of apoptosis. Our data (**Figure 4D**) show that the combination of GDC-0980 plus T-DM1 significantly induced mitochondrial depolarization via upregulation of cleaved CASPASE 3, cleaved PARP, and BIM (please see the Western blot data in **Figure 4B**). In BT474 cells, a single-agent TD-M1 had limited effect on the depolarization while the combination induced mitochondrial depolarization at 48 hours **Figure 4Di**. In contrast, single-agent TD-M1 had a pronounced effect on depolarization, and the combination was effective at lower doses in HCC1954 **Figure 4Dii**.

GDC-0980-mediated alterations of PI3K and MAPK pathways

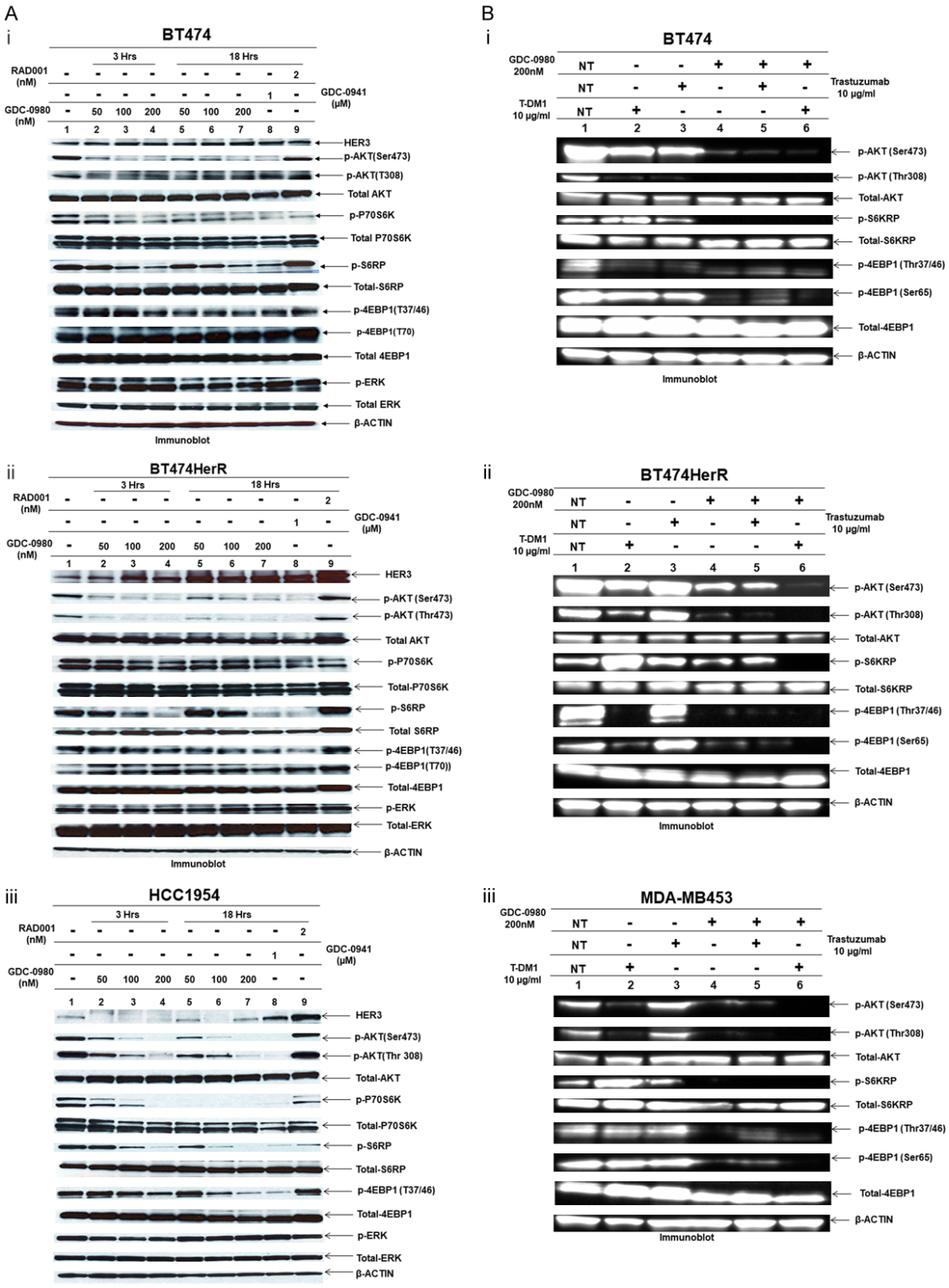
To investigate potential molecular markers of response to GDC-0980, we examined the signaling components of the PI3K-mTOR signaling network, including RAS-MAPK in cell lines representing T-sensitive (BT474), T-resistant (BT474HerR), activating mutation of *PIK3CA* (HCC1954, MDA-MD453) and *PTEN* mutated (HCC1569). In each cell line treated with GDC-0980 (indicated concentration and time), phosphorylation of downstream markers of the PI3K-AKT-mTOR pathway was reduced (**Figure 2Ai-iv**). These included signaling markers downstream of PI3K, such as phospho-AKT (Thr308), which is phosphorylated by PDK1, biomarkers downstream of mTORC2 such as phospho-AKT (Ser473), and biomarkers downstream of mTORC1 such as phospho-P70S6K, phospho-S6 ribosomal protein (S6RP), and p-4EBP1 (Thr 37/46 and Thr70) (**Figure 2Ai-iv**). We also assessed phospho-ERK (a downstream marker of the RAS-MAPK pathway) in GDC-0980 treated cells and observed no significant alterations in all four cell lines. The upregulation of HER3 expression was observed only in T-resistant cells (BT474HerR, uppermost panel) (**Figure 2Aii**). Conversely, RAD001-induced significant upregulation of HER3 was observed in both T-resistant (lane 9) and *PIK3CA* mutated cells (HCC1954, lane 9) (**Figure 2Aii**, **2Aiii**). Interestingly, HER3 upregulation was also observed in a higher time point following the treatment of a pan-PI3K treatment (GDC-0941) in HCC1954 cells (lane 8) (**Figure 2Aiii**). Like 3D-ON-TOP proliferation studies, the combination of GDC-0980 and trastuzumab or T-DM1 was more effective in all cells irrespective of *PIK3CA* mutation or not (**Figure 2Bi-iii**). Interestingly, our data also demonstrated that both trastuzumab and T-DM1 significantly induced the expression of PD-L1 (one of the important biomarkers for immunotherapy) under hypoxic conditions in *HER2* amplified BT474 cells (**Figure 3**).

GDC-0980 induces apoptosis in HER2+ breast cancer cell lines

Consistent with the anti-clonogenic effects of GDC-0980 (**Figure 1**), GDC-0980 induced apoptosis in HER2+ breast cancer cells. We assessed AnnexinV staining, an early marker of apoptosis induction by flow cytometry, in addition to biochemical markers of apoptosis (cleaved Caspase3 and cleaved PARP) by Western blotting in T-sensitive, T-resistant, and *PIK3CA* mutated/HER2+ cell lines (**Figure 4A-C**). The combination of T-DM1 plus GDC-0980 was more effective in the induction of apoptosis in both BR474 (**Figure 4Ci**) and HCC1954 (**Figure 4Cii**) cells. Moreover, we also determined the real-time changes in the number of apoptotic cells following the treatment of GDC-0980 at 48 hours. Apoptotic cells were determined by real-time counting of Cytox green positive cells in IncuCyte Zoom (Live Cell Imaging Incubator, Essen BioScience) treated with 500 nM GDC-0980 (**Figure 4Ci**, **4Cii**) and, bottom panels). Our data (flow cytometry, biochemical and real-time IncuCyte) suggest that PI3K and AKT inhibition are key molecules of this pathway for the induction of apoptosis by GDC-0980 treatment and that mTORC2 (and its immediate target effector, i.e., serine phosphorylated AKT) more than mTORC1 is probably involved in this event (since RAD-001 has little to no effect on AnnexinV staining) (data not shown).

Anti-tumor efficacy of GDC-0980, trastuzumab, T-DM1, or in combination

Evolutionarily conserved signaling pathways are often characterized by their inherent plasticity. Thus a complete inhibition of signaling pathways usually requires combinatorial therapies. Aberrant activation of the PI3K-AKT-mTOR pathway has been shown to correlate with a


A
i
BT474
3 Hrs
18 Hrs
RAD001 (nM)
GDC-0941 (μM)
GDC-0980 (nM)
HER3
p-AKT(Ser473)
p-AKT(T308)
Total AKT
p-P70S6K
Total P70S6K
p-S6RP
Total-S6RP
p-4EBP1(T37/46)
p-4EBP1(T70)
Total 4EBP1
p-ERK
Total ERK
β-ACTIN
Immunoblot
ii
BT474HerR
p-AKT (Ser473)
p-AKT (Thr473)
Total-AKT
p-P70S6K
Total-P70S6K
p-S6RP
Total S6RP
p-4EBP1(T37/46)
p-4EBP1(T70))
Total-4EBP1
p-ERK
Total-ERK
β-ACTIN
Immunoblot
iii
HCC1954
HER3
p-AKT(Ser473)
p-AKT(Thr 308)
Total-AKT
p-P70S6K
Total-P70S6K
p-S6RP
Total-S6RP
p-4EBP1 (T37/46)
Total-4EBP1
p-ERK
Total-ERK
β-ACTIN
B
i
BT474
GDC-0980 200nM
T-DM1 10 μg/ml
Trastuzumab 10 μg/ml
NT
p-AKT (Ser473)
p-AKT (Thr308)
Total-AKT
p-S6KRP
Total-S6KRP
p-4EBP1 (Thr37/46)
p-4EBP1 (Ser65)
Total-4EBP1
β-ACTIN
Immunoblot
ii
BT474HerR
iii
MDA-MB453

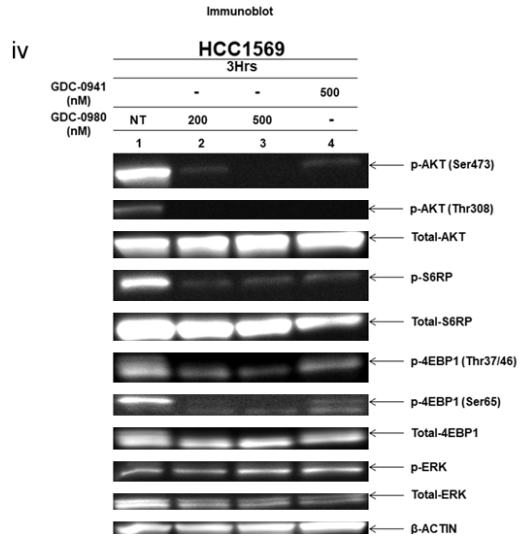


Figure 2. Effects of GDC-0980 (A) and a combination of GDC-0980 plus trastuzumab/T-DM1 (B) in HER2+ breast cancer cells: Western blot showing GDC-0980 time/dose-dependently blocked activation of AKT and its downstream effectors in total lysates from HER2+ [trastuzumab-sensitive BT474 (Ai), trastuzumab-resistant BT474HerR (Aii), *PIK3CA* mutated HCC1954 (Aiii) and *PTEN* mutated HCC1569 (Aiv) breast cancer cells. Time-course experiments revealed that at both time points (3 hrs and 18 hrs), GDC-0980 significantly blocked phosphorylation of AKT at ser 473 and the 308 as well as downstream effectors of mTOR (p-P70S6K, p-S6RP, and p-4EBP1). No significant alterations of p-ERK were observed in all four cell lines. When compared with GDC-0941 (pan-PI3K inhibitor) (lane 8) and RAD001 (mTORC1 inhibitor) (lane 9), GDC-0980 more significantly abrogated the PI3K-AKT-mTOR signaling. The upregulation of HER3 expression was observed only in T-resistant cells (BT474HerR, uppermost panel) (Aii). Conversely, RAD001-induced significant upregulation of HER3 was observed in both T-resistant (lane 9) and *PIK3CA* mutated cells (HCC1954, lane 9) (Aii and Aiii). Interestingly, HER3 upregulation was also observed in a higher time point following the treatment of a pan-PI3K treatment (GDC-0941) in HCC1954 cells (lane 8) (Aiii). Moreover, the PI3K-AKT-mTOR pathway inhibition was more prominent when trastuzumab (10 μg/ml) or T-DM1 (10 μg/ml) was treated along with GDC-0980. In all three cell lines (BT474, BT474HerR, and MDA-MB453), single-agent T-DM1 significantly induced pS6RP (Bi-Biii, lane 2, row 4), and differential inhibition of p-4EBP1 was also observed. T-DM1 significantly abrogated p-4EBP1 in BT474HerR (Bii lane 2 and row 6 & 7) cells but failed to inhibit in MDA-MB453 cells (Biii lane 2 and row 6 & 7). We are unable to explain this differential inhibition of p-4EBP1 following the treatment of T-DM1.

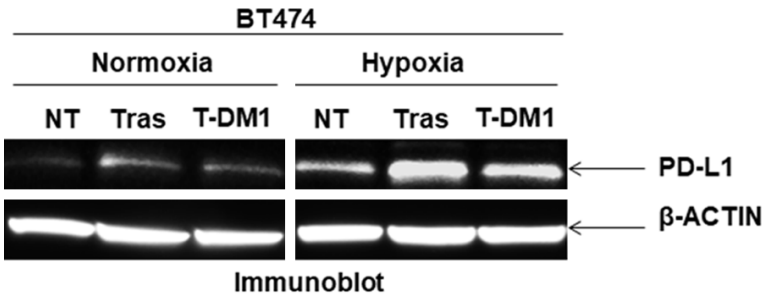


Figure 3. Trastuzumab/T-DM1 induces PD-L1 expression in *HER2* amplified breast cancer cells. BT474 cells were treated with 10 μg/ml trastuzumab or T-DM1 and incubated either at normoxic or in hypoxic conditions for 4 hours. Data showed higher expression of PD-L1 following the treatment in both normoxic and hypoxic conditions, although the expression was significantly higher in hypoxic conditions. This result provides a rationale for testing/initiating a clinical trial combining an anti-HER2 agent along with immunotherapy in *HER2*+ breast cancer patients.

diminished response to HER2 directed therapies [44]. Approximately 30%-40% of HER2+ breast cancers harbor an activating mutation of PIK3CA [45], consistent with the notion that these two oncogenes have non-overlapping functions and may cooperate to promote tumorigenesis [46].

Several studies suggest that activation of the PI3K pathway and/or activating mutations of PIK3CA confer resistance to anti-HER2 therapy including trastuzumab [44, 47, 48], however confirmation of a causal relationship between the aberrant relationship of the PI3K-AKT-mTOR pathway and resistance to trastuzumab in the clinic is not clear yet. Here we sought to examine

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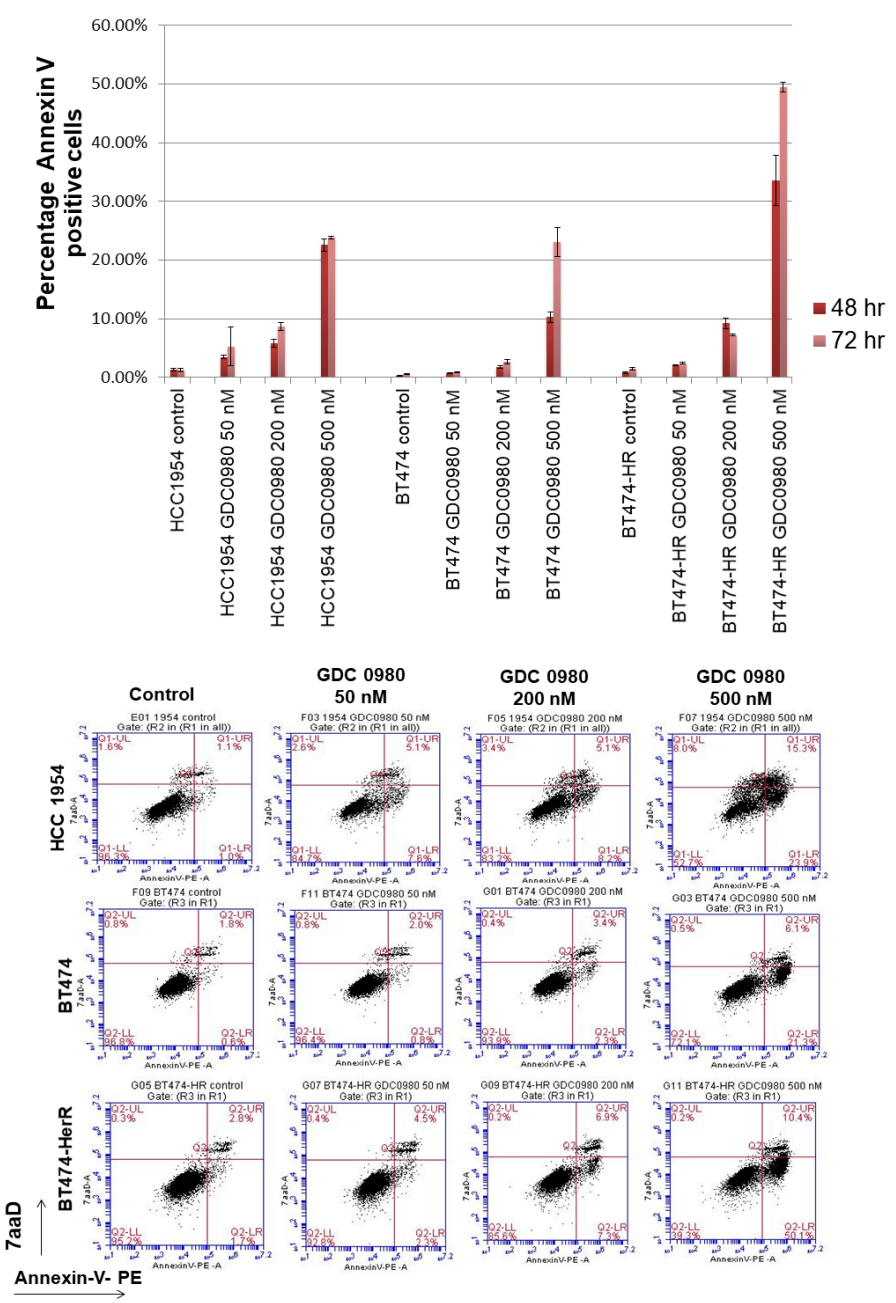
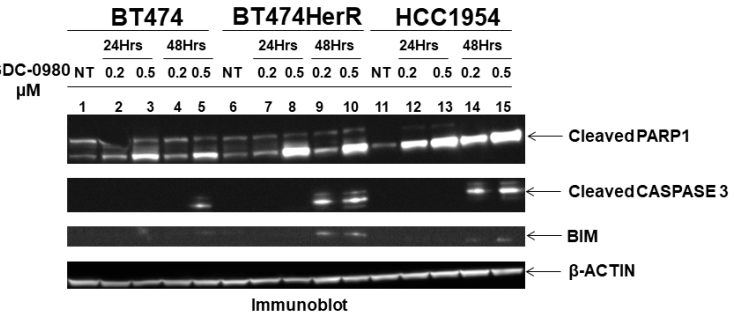
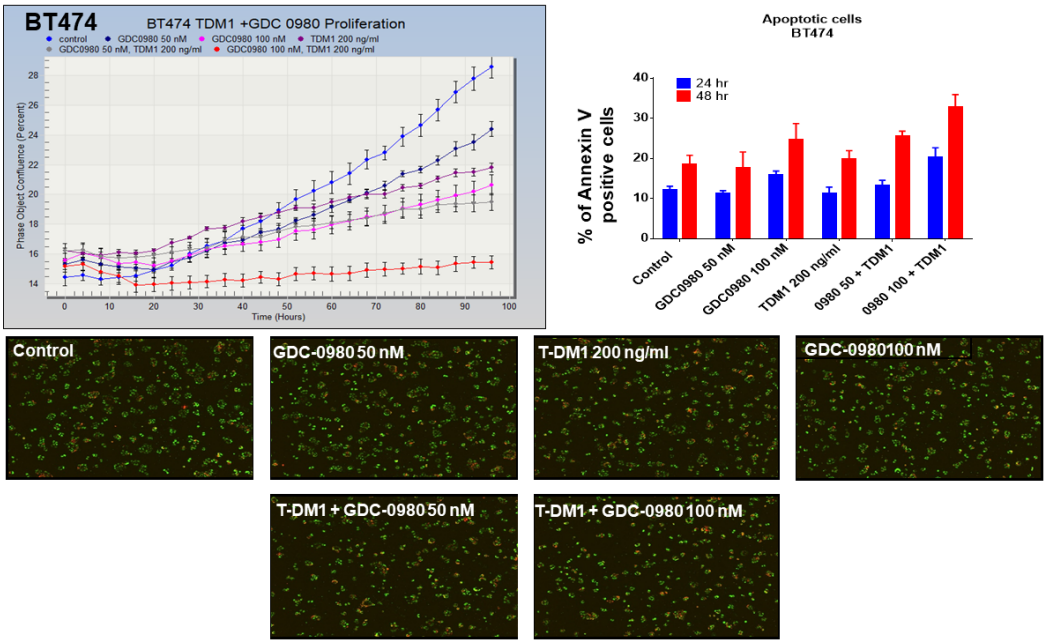

Percentage Annexin V positive cells
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
48 hr
72 hr
HCC1954 control
HCC1954 GDC0980 50 nM
HCC1954 GDC0980 200 nM
HCC1954 GDC0980 500 nM
BT474 control
BT474 GDC0980 50 nM
BT474 GDC0980 200 nM
BT474 GDC0980 500 nM
BT474-HR control
BT474-HR GDC0980 50 nM
BT474-HR GDC0980 200 nM
BT474-HR GDC0980 500 nM
Control
GDC 0980 50 nM
GDC 0980 200 nM
GDC 0980 500 nM
HCC 1954
BT474
BT474-HerR
7aaD
Annexin-V- PE

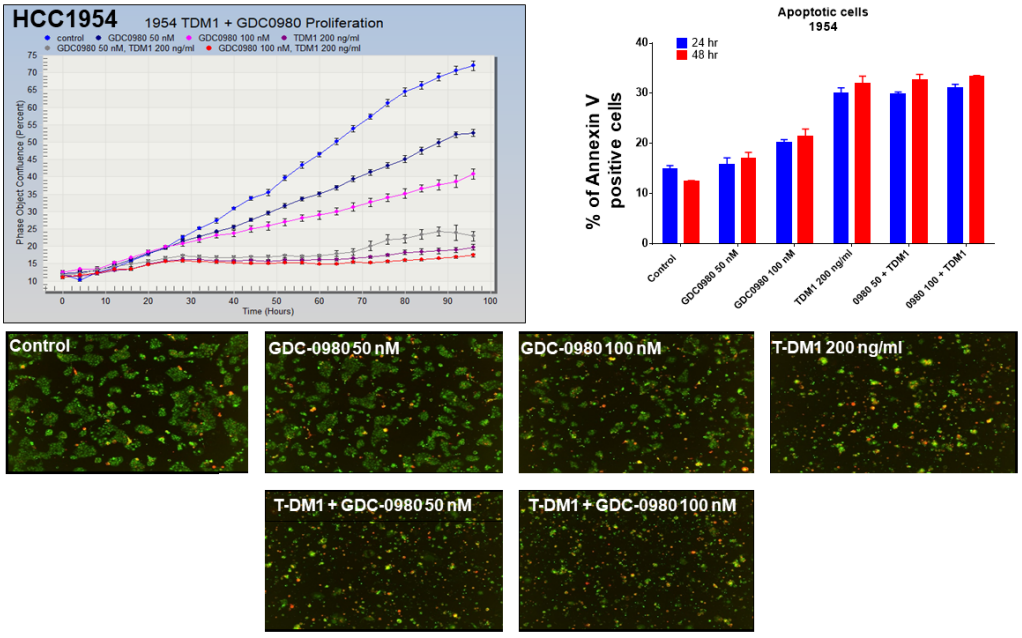
B


BT474
BT474HerR
HCC1954
24Hrs
48Hrs
GDC-0980 μM
NT 0.2 0.5 0.2 0.5 NT 0.2 0.5 0.2 0.5 NT 0.2 0.5 0.2 0.5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Cleaved PARP1
Cleaved CASPASE 3
BIM
β-ACTIN
Immunoblot

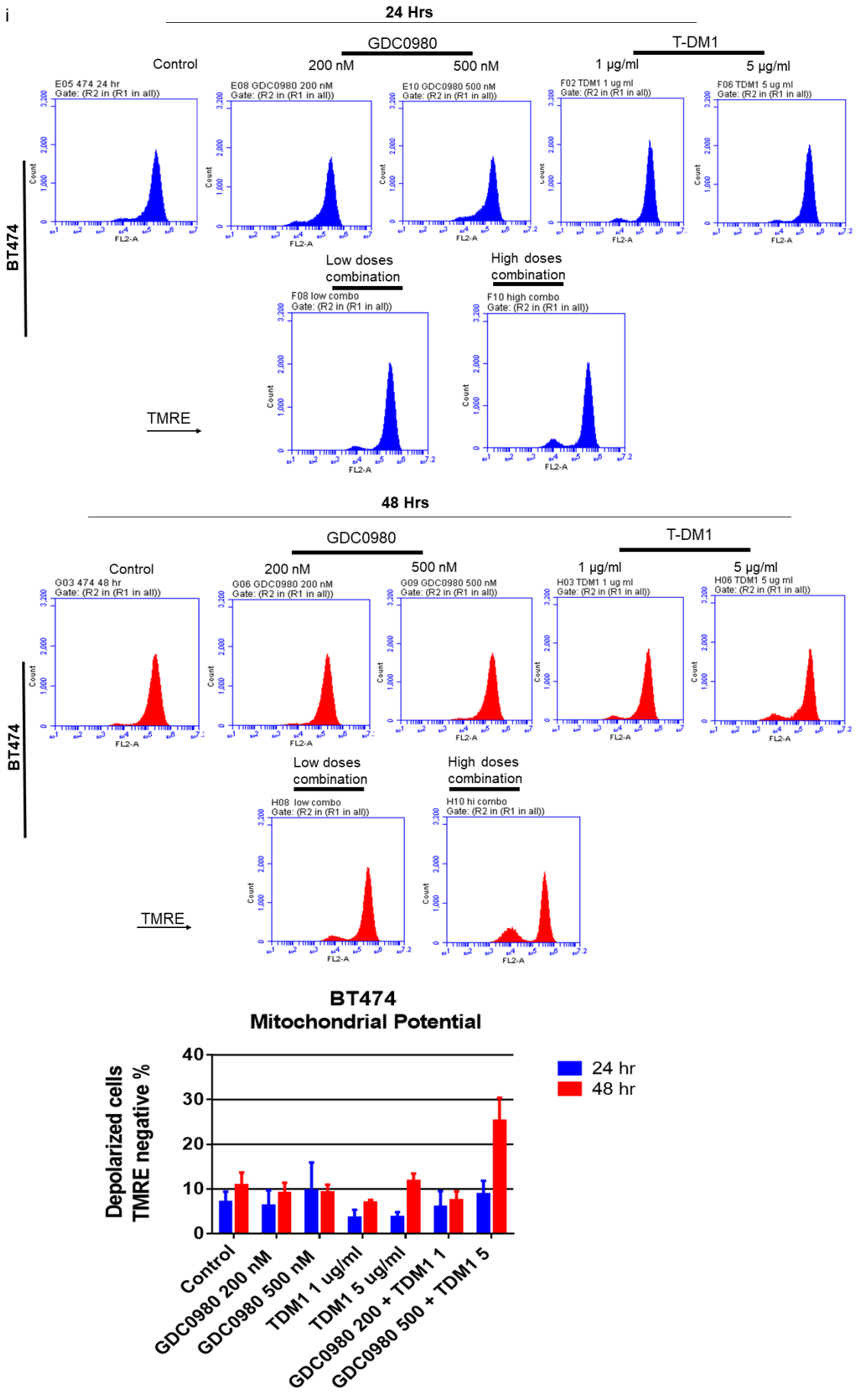
C i


BT474
BT474 TDM1 +GDC 0980 Proliferation
control
GDC0980 50 nM
GDC0980 100 nM
TDM1 200 ng/ml
GDC0980 50 nM, TDM1 200 ng/ml
GDC0980 100 nM, TDM1 200 ng/ml
Phase Object Confluence (Percent)
Time (Hours)
Apoptotic cells
BT474
24 hr
48 hr
% of Annexin V positive cells
Control
GDC0980 50 nM
GDC0980 100 nM
TDM1 200 ng/ml
0980 50 + TDM1
0980 100 + TDM1
Control
GDC-0980 50 nM
T-DM1 200 ng/ml
GDC-0980100 nM
T-DM1 + GDC-0980 50 nM
T-DM1 + GDC-0980 100 nM

ii


HCC1954
1954 TDM1 + GDC0980 Proliferation
control
GDC0980 50 nM
GDC0980 100 nM
TDM1 200 ng/ml
GDC0980 50 nM, TDM1 200 ng/ml
GDC0980 100 nM, TDM1 200 ng/ml
Phase Object Confluence (Percent)
Time (Hours)
Apoptotic cells
1954
24 hr
48 hr
% of Annexin V positive cells
Control
GDC0980 50 nM
GDC0980 100 nM
TDM1 200 ng/ml
0980 50 + TDM1
0980 100 + TDM1
Control
GDC-0980 50 nM
GDC-0980 100 nM
T-DM1 200 ng/ml
T-DM1 + GDC-0980 50 nM
T-DM1 + GDC-0980 100 nM

D i


24 Hrs
GDC0980
T-DM1
Control
200 nM
500 nM
1 µg/ml
5 µg/ml
BT474
Low doses combination
High doses combination
TMRE
48 Hrs
BT474
Mitochondrial Potential
Depolarized cells TMRE negative %
24 hr
48 hr
Control
GDC0980 200 nM
GDC0980 500 nM
TDM1 1 ug/ml
TDM1 5 ug/ml
GDC0980 200 + TDM1 1
GDC0980 500 + TDM1 5

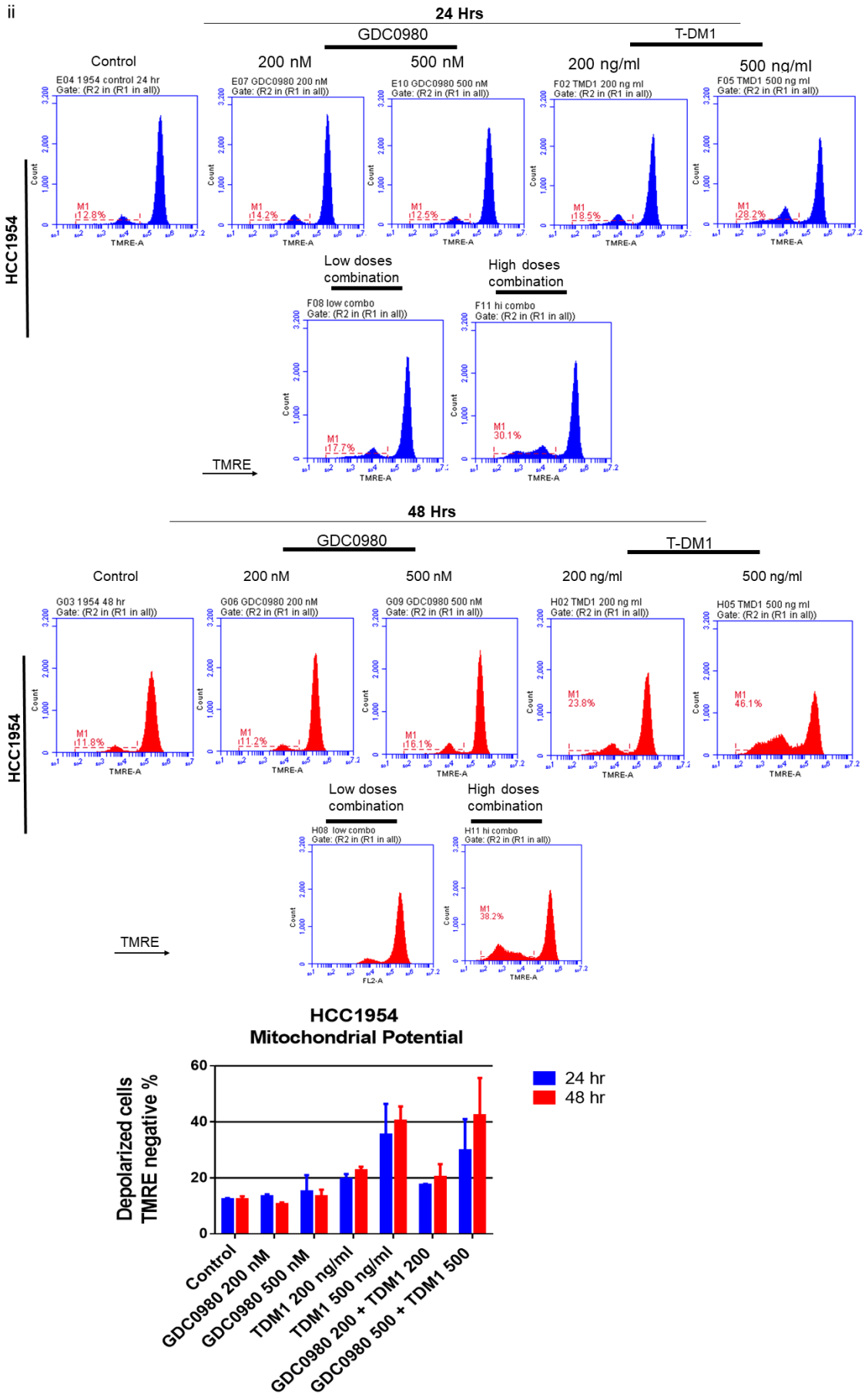

ii
24 Hrs
GDC0980
T-DM1
Control
200 nM
500 nM
200 ng/ml
500 ng/ml
HCC1954
Low doses combination
High doses combination
TMRE
48 Hrs
HCC1954
Mitochondrial Potential
Depolarized cells
TMRE negative %
24 hr
48 hr
Control
GDC0980 200 nM
GDC0980 500 nM
TDM1 200 ng/ml
TDM1 500 ng/ml
GDC0980 200 + TDM1 200
GDC0980 500 + TDM1 500

Figure 4. Effects of GDC-0980 on the induction of apoptosis: (A) Dose-dependent effect of GDC-0980 (50, 200, and 500 nM) on apoptotic (early) response analyzed by annexin V/7AAD staining in BT474, BT474HerR, and HCC1954. Cells were treated for 48 hours, released, rinsed, and placed in the annexin V binding buffer. Cells were labeled with annexin V-PE and 7AAD for analysis. Error bars represent SEM from triplicates. The number of annexin-V positive cells (an indicator of apoptosis) was dose-dependently increased in both 48 and 72 hours of drug exposure (Bar-diagram is presented in the Upper Panel & Histogram is presented in the Lower Panel). (B) GDC-0980 induces apoptosis through the induction of cleaved CASPASE3, cleaved PARP1, and induction of BCL-2 family protein BIM that promotes apoptosis in all three indicated cell clines. Cell lines were incubated for different time periods (24 and 48 hours) with the indicated amount of GDC-0980. Data showed that cleaved PARP1 and cleaved CASPASE3 expression were more at higher concentrations of GDC-0980. Furthermore, the expression was more in BT474HerR and HCC1954 cells might be due to addiction of the PI3K-AKT-mTOR pathway in those cell lines. BIM expression was low but only expressed at a higher time point in BT474HerR (lane 9, 10) and HCC1954 (lane 15) cells. Beta-ACTIN was used as a loading control. (C) In separate experiments, the effect of single-agent GDC-0980, T-DM1, and a combination of GDC-0980 plus T-DM1 in BT474 (i) and HCC1954 (ii) cells were tested by real-time proliferation (upper left panel), apoptosis (upper right panel), and staining of live-dead cells (lower panel). Data clearly indicate that GDC-0980 plus T-DM1 significantly blocks cell proliferation, induces apoptosis, and also increases dead cells (red in color) staining compared to either agent alone. (D) Mitochondrial Potential was measured using 400 nM TMRE (tetramethylrhodamine, ethyl ester) staining performed in triplicate, and a representative image is shown. As a control for depolarization, cells were briefly treated with 20 μM FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone) before adding TMRE. (Abcam, Cambridge, United Kingdom) Cells were collected on a BD Accuri C6 flow cytometer. Here we validated the mitochondrial involvement in inducing apoptosis by TMRE-based mitochondrial membrane potential under the same treatment condition cell lines BT474 (i, Upper Panel represents 24 hours time point. Middle Panel represents 48 hours time point. Lower Panel represents the Bar-diagram of both time points) and HCC1954 (ii, Upper Panel represents 24 hours time point. Middle Panel represents 48 hours time point. Lower Panel represents the Bar-diagram of both time points).

whether the combination of a dual PI3K/mTOR inhibitor GDC-0980 along with trastuzumab or T-DM1 may effectively override the PI3K pathway-mediated trastuzumab resistance in a mouse xenograft model. The in vitro sensitivity profile of GDC-0980 alone or a combination of trastuzumab or T-DM1 plus GDC-0980 was recapitulated in *in vivo* in BT474 (**Figure 5A**) and BT474HerR xenograft models (**Figure 5C**). In both T-sensitive and resistant models [BT474HerR (**Figure 5C**) and HCC1954 (*PIK3CA* mutated) (**Figure 5E**)], a combination treatment of GDC-0980 plus trastuzumab or T-DM1 achieved a more effective blockade of the tumor growth than either drug in isolation (**Figure 5**), suggesting that inhibition of both oncogenic signals are required to inhibit tumor growth to tumor regression (**Figure 5G**). Compared to single-agent (either GDC-0980 or trastuzumab or T-DM1) or a combination of GDC-0980 plus trastuzumab treatments, the combination treatment of GDC-0980 plus T-DM1 significantly enhanced tumor growth inhibition in all xenograft models (**Figure 5**). Our study reveals the preclinical evidence in favor of the inclusion of T-DM1 instead of trastuzumab when combined with GDC-0980 in concurrent alterations of *HER2+* amplification and *PIK3C*A mutation in breast cancer patients.

To further investigate the mechanism of action of GDC-0980 alone or its combination with trastuzumab or T-DM1 in tumor growth inhibition, expression levels of the proliferative marker (Ki67), tumor angiogenic markers (CD31 and p-VEGFR2), and PI3K-AKT-mTOR signaling pathway-specific PD markers (p-S6RP) were determined by IHC from the FFPE sections of the tumors from animals from each arm of the xenograft study. High expression of Ki67 has been associated with poorer outcomes, and measurement of Ki67 index pre-and post-therapy provided an accurate surrogate for the responsiveness of breast cancer to the treatment [49]. GDC-0980 treated tumors showed a significant decrease in micro-vessel density (MVD) (positive for CD31 and positive for p-VEGFR2 IHC staining), suggesting that this treatment has substantial tumor-induced anti-angiogenic properties in vivo. PD studies showed a decrease in the Ki67, CD31, p-VEGFR2, and the PI3K pathway-specific markers, like the p-S6RP expression in tumors from mice treated with GDC-0980 in combination with trastuzumab or T-DM1 as compared to the control (**Figure 5B**, **5D** and **5F**). Our *in vitro* data is in line with the xenograft data, which showed that GDC-0980, in combination with trastuzumab or T-DM1, was an efficacious anti-tumor drug combination in HER2+/T-resistant as well as HER2+/*PIK3CA*-mutated breast cancer models.

Discussion

There is an unmet need for novel pathway-specific target therapeutics that are more precisely

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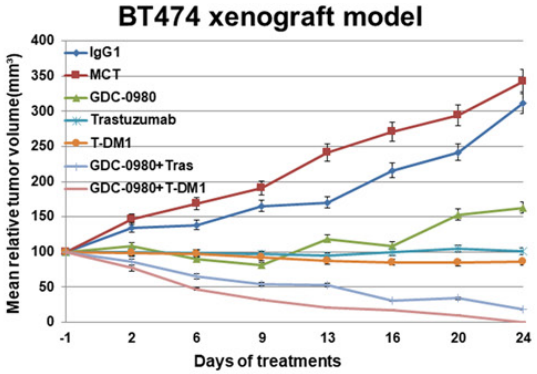
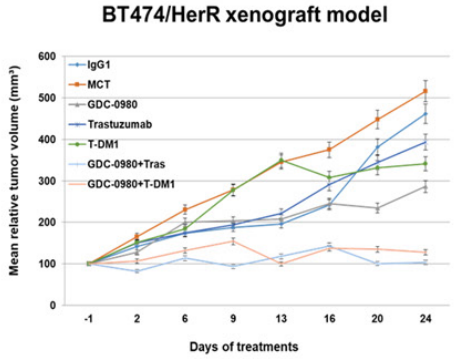


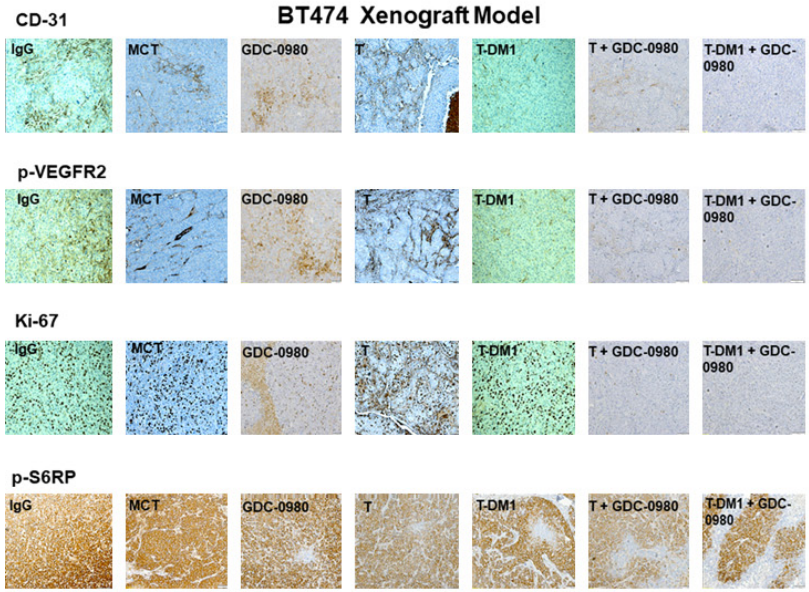
Arms of treatments	BT474 xenograft model Tumor volume (mm³ ± SEM)	Significant (*p* value)	Body weight (gram ± SEM)
1. Ctrl. IgG1	890 ± 244		23.4 ± 0.4
2. Ctrl. MCT (for GDC-0980)	691 ± 211		23.3 ± 0.5
3. GDC-0980 10mg/kg, oral gavage, once daily.	237 ± 29	$p < 0.00094$	24.4 ± 0.2
4. Trastuzumab 10 mg/kg in 100 µl, i.p. twice weekly	119 ± 9	$p < 0.00041$	25.1 ± 0.8
5. T-DM1 10 mg/kg in 100 µl, i.v. every 3 week	147 ± 21	$p < 0.00084$	22.2 ± 0.1
6. GDC-0980 + Tras	28 ± 8	$p < 0.00018$	23.7 ± 0.3
7. GDC-0980 + T-DM1	0 ± 0	$p < 0.000094$	23.9 ± 0.3

C



Arms of treatments	BT474/HerR xenograft model Tumor volume (mm³ ± SEM)	Significant (*p* value)	Body weight (gram ± SEM)
1. Ctrl. IgG1	832.3 ± 390		23.8 ± 1.20
2. Ctrl. MCT (for GDC-0980)	1126 ± 184		24.4 ± 0.20
3. GDC-0980 10mg/kg, oral gavage, once daily.	382.8 ± 56.7	$p < 0.00362$	23.9 ± 0.18
4. Trastuzumab 10 mg/kg in 100 µl, i.p. twice weekly	630 ± 246	$p < 0.01755$	25.7 ± 0.50
5. T-DM1 10 mg/kg in 100 µl, i.v. every 3 week	518 ± 63.9	$p < 0.02026$	20.4 ± 0.42
6. GDC-0980 + Tras	143.2 ± 26.2	$p < 0.00096$	23.7 ± 0.21
7. GDC-0980 + T-DM1	182.8 ± 37.5	$p < 0.00132$	25.0 ± 0.55

B



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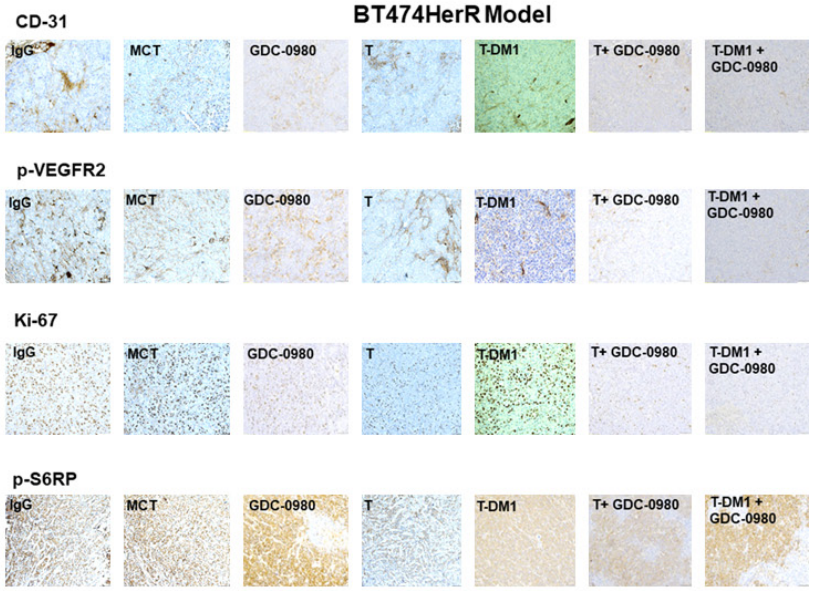

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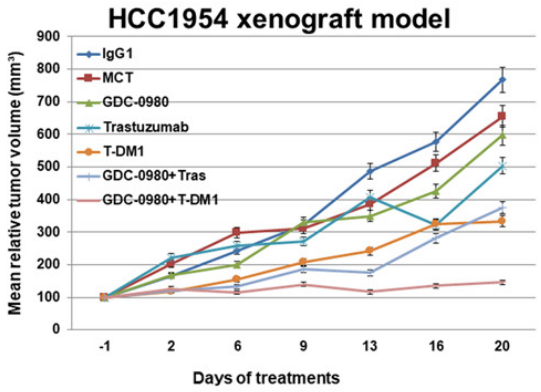
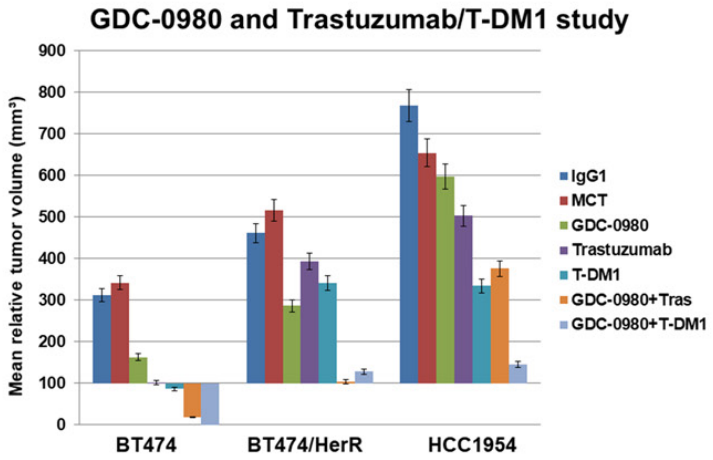


Arms of treatments	HCC1954 xenograft model		
	Tumor volume (mm³ ± SEM)	Significant (*p* value)	Body weight (gram ± SEM)
1. Ctrl. IgG1	1330 ± 16		24.4 ± 0.2
2. Ctrl. MCT (for GDC-0980)	1369 ± 121		23.8 ± 0.2
3. GDC-0980 10mg/kg, oral gavage, once daily.	965 ± 103	$p < 0.168$	23.9 ± 0.1
4. Trastuzumab 10 mg/kg in 100 µl, i.p. twice weekly	1121 ± 127	$p < 0.489$	23.1 ± 0.3
5. T-DM1 10 mg/kg in 100 µl, i.v. every 3 week	561 ± 48	$p < 0.055$	23.2 ± 0.2
6. GDC-0980 + Tras	601 ± 114	$p < 0.038$	22.9 ± 0.3
7. GDC-0980 + T-DM1	234 ± 16	$p < 0.013$	22.9 ± 0.3

G



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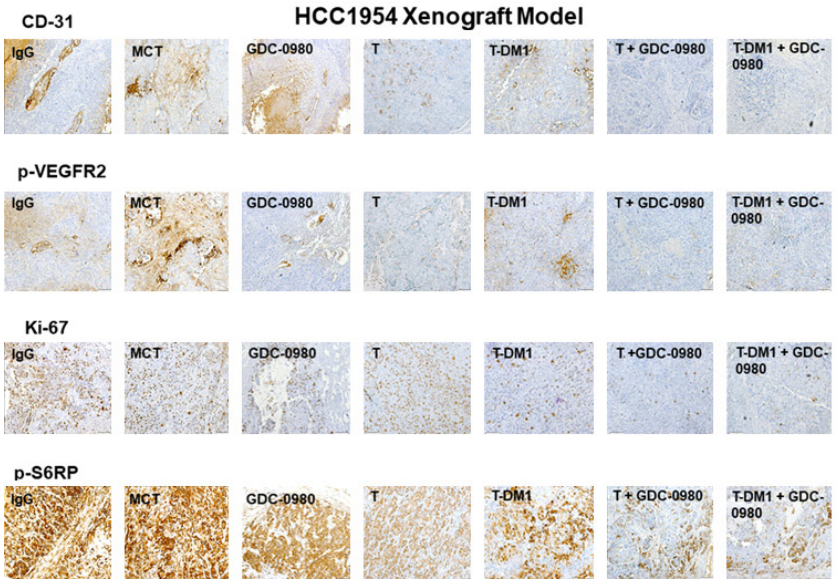

Figure 5. Efficacy of GDC-0980, trastuzumab, T-DM1 or in a combination of GDC-0980 trastuzumab or GDC-0980 plus T-DM1 in BT474 (A, B), BT474HerR (C, D), and HCC1954 (E, F) human tumor xenograft models: A pilot study was conducted with HCC1954 cells to determine 1) the number of cells required to inject for the establishment of tumors and their maintenance in animals throughout drug administration and 2) the maximum tolerable dose of drugs in animals with tumor burden. The number of cells injected was adjusted based on the tolerable tumor burden in untreated animals (following IACUC guidelines). Based on the results of the pilot study, cells were injected in matrigel subcutaneously into the flank of immunocompromised female nude (nu/nu) mice. Established xenograft tumors were treated with GDC-0980 (10 mg/kg, oral, once-daily) or trastuzumab (10 mg/kg, i.p., twice weekly) or T-DM1 (10 mg/kg i.v. every three weeks) and in a combination of GDC-0980 plus trastuzumab or GDC-0980 plus T-DM1. The table [(lower panel of (A, C and E)] shows the change in volumes of xenograft tumors and body weights of the mice in response to drug(s) treatment. PD data of BT474 tumor (B), BT474HerR (D), and HCC1954 (F) for cell proliferation marker (Ki67), tumor-induced angiogenic markers (CD31 & pVEGFR and cell signaling markers (pS6RP) were presented. (G) A dual PI3K/mTOR inhibitor (GDC-0980) plus trastuzumab or GDC-0980 plus T-DM1 showed *in vivo* efficacy in mouse xenograft models. Quantification of the mean changes (relative values) in tumor volume of xenografts after 3 weeks of treatment, negative values indicate tumor regression.

adapted to the molecular era of genomic-driven medicine. Aberrant activation of the PI3K-AKT-mTOR pathway has been reported to reduce the efficiency of trastuzumab-based therapy in HER2+ BC patients. The PI3K-AKT-mTOR pathway plays a critical role in cellular growth, survival, migration/invasion, and angiogenesis, and aberrations in this signaling pathway have been concerned in the occurrence of many cancers, including breast cancer. It has been proposed that the complex crosstalk between intracellular signaling pathways, is often responsible for the development of the resistance following a drug-therapy. It has been reported by others that PI3K inhibitor failed to induce substantial apoptosis in ER+ breast cancer cells, although they exhibited suppression of AKT phosphorylation upon treatment with a PI3K inhibitor. However, phosphorylation of S6RP (immediately downstream of mTOR) was maintained [50, 51]. These data suggest that the sensitivity to PI3K inhibitor, even in *PIK3CA* mutated cells, maybe not sufficient to block mTOR and its downstream signaling. Targeting multiple nodes of a single pathway may improve response to therapy. One of the key advantages of a dual, PI3K and mTOR inhibitor, such as GDC-0980, over other isoform-specific PI3K inhibitors, is the potential to treat tumors harboring oncogenic abnormalities that signal through different PI3K isoforms and also mTOR as well as its downstream effector molecules, which are activated by upstream PI3K-dependent and independent manners. Inhibition of PI3K could abrogate the feedback induction that results from mTORC1 inhibition and mTOR inhibition more efficiently blocked distal PI3K signaling at the level of S6 ribosomal protein or 4EBP1 that is not sufficiently inhibited by PI3K inhibition alone. Breast cancers are clear disease targets for the PI3K-mTOR pathway inhibitor use [52]. The most common modes of PI3K activation in breast cancer include amplification of the human ERBB2 gene (HER2), which encodes a receptor tyrosine kinase that interacts with PI3K [14]; activating mutations in the PIK3CA gene, which encodes p110α, the catalytic subunit of PI3K (25 to 30%) [53, 54]; and loss of the phosphatase and tensin (PTEN) tumor suppressor gene, which encodes a protein phosphatase and lipid phosphatase that negatively regulates PI3K-AKT signaling pathways (10%) [45, 55]. Earlier, Sarat Chandarlapaty and the group reported that the combined rate of PTEN loss and PIK3CA mutation in the trastuzumab-resistant tumors was 71% [56]. A retrospective study in HER2-positive metastatic breast cancer (MBC) (n = 47) showed that PTEN mutation and overexpression of the PI3K catalytic subunit were both associated with reduced progression-free survival (PFS), and the latter was found to independently predict time to progression (TTP) or death (hazard ratio [HR]: 2.75; 95% CI: 1.14-6.49; P: 0.024) [57]. Hence, this combination is expected to be most active in tumors with the PI3K pathway addiction resulting from 1) HER2 overexpression-induced HER2: HER3 heterodimerization, 2) mutations in genes such as PTEN or PIK3CA, and 3) in other lesions where RAS-MAPK signaling is not constitutively active.

We have recently published that targeting the PI3K-AKT-mTOR signaling pathway has antitumor efficacy in combination with using trastuzumab [58]. Our preclinical data show that targeting two different nodal points (PI3K and mTOR) of the PI3K pathway is highly efficacious along with T-DM1 in T-sensitive, T-resistant, and HER2/PIK3CA co-amplified breast cancer models. This is clinically significant, as resistance to trastuzumab therapy is a common clinical problem that limits the survival in patients with HER2 amplified breast cancer and activation of the PI3K-AKT-mTOR pathway lying downstream of HER2 and HER3, which facilitates signaling independently of the HER2 kinase, plays an essential role for the development of *de novo* and acquired resistance to trastuzumab therapy. As agents targeting PI3K/mTOR, such as BEZ235 or GDC-0980, are currently undergoing clinical investigation, these data would support the combination of the PI3K-AKT-mTOR pathway targeted therapy with trastuzumab or T-DM1 in HER2+ breast cancer.

GDC-0980 treatment resulted in survival changes measured by 3D-ON-TOP clonogenic growth (including heregulin-induced clonogenic growth) (**Figure 1**), real-time cell proliferation (**Figure 4C**), and apoptotic markers (**Figure 4A**, **4B**). At the same time, apoptosis was not observed with RAD001 (everolimus, an allosteric inhibitor of mTOR) (data not shown), indicating that either PI3K and/or mTORC2 is sufficient for cell survival. Mitochondrial depolarization data indicates that the presence of *PIK3CA*

mutation at H1047R in the absence of estrogen receptor is making the HCC1954 cells more sensitive to the combination (TD-M1 plus GDC-0980) as compared to estrogen receptor-positive BT474 cells (**Figure 4D**). Despite some success in selected tumor types, rapalogs generally showed limited anti-cancer efficacy as a single agent [59]. In the same line, both BOLERO-1 and BOLERO-3 studies showed limited success with everolimus was used with trastuzumab in HER2+ advanced breast cancer patients [31, 32, 60]. One of the important reasons is a termination of a negative feedback loop involving S6K to upstream receptor tyrosine kinase and its *bonafide* adapter protein. Targeting tumors with rapalogs can result in increased PI3K-AKT activity leading to an enhanced proliferation rate of the tumor and decreased the efficacy of allosteric mTOR inhibitors [33]. ATP competitive mTORC1/C2 kinase inhibitors (e.g., OSI-027, AZD2014, and TAK228) have been developed and are undergoing clinical trials. However, resistance to these mTOR kinase inhibitors can still arise via the PI3K feedback mechanism, by increased p-AKT at threonine 308 or activation of AKT-independent of PI3K targets or some activating mutations of mTOR either its FRB domain or its kinase domain [61, 62]. Moreover, upregulation of the RAS-MAPK pathway that occurs following mTORC1 inhibition alone following the treatment of everolimus as well as inhibition of PI3K leads to compensatory activation of upstream receptor tyrosine kinases that limit the effectiveness of those compounds [63-65]. We have previously demonstrated that either BEZ235 or GDC-0980 has profound anti-tumor efficacy when combined with trastuzumab or veliparib (a PARP inhibitor) in HER2+ or BRCA competent triple-negative breast cancer model respectively [38, 58]. Our data demonstrated that dual PI3K/mTOR inhibition caused sustained inhibition of AKT, p70S6K, S6RP, and 4EBPs. Differing from the report by Choo and Blenis [66] and Choo et al. [67], who showed that with rapamycin, after an initial inhibitory effect on 4EBP1 phosphorylation, phosphorylation re-appeared, we observed a complete inhibition of p4EBP1 in *PIK3CA* mutated (HCC1954) (**Figure 2Aiii**) and *PTEN* null (HCC1569) (**Figure 2Aiv**) *HER2* amplified breast cancer cells. Importantly, inhibition was more potent when trastuzumab or T-DM1 was combined with a low dosage of GDC-0980 (200 nM) in trastuzumab-sensitive, BT474, trastuzumab-resistant BT474HerR, and *PIK3CA* mutated *HER2* amplified MDA-MB-453 cells (**Figure 2Bi-iii**). In all cell lines, we have observed that as a single agent T-DM1 significantly induced pS6RP (**Figure 2Bi-iii**, lane 2, row 4), and differential inhibition of p4EBP1 expression. T-DM1 significantly abrogated p4EBP1 in BT474HerR (**Figure 2Bii** lane 2 and row 6 & 7) cells but failed to inhibit in MDA-MB453 cells (**Figure 2Biii** lane 2 and row 6 & 7). At this moment, it is difficult to explain the reason why T-DM1 would have different activities in terms of mTOR signaling. More studies are needed to reveal this mechanism. More importantly, a combination of trastuzumab plus GDC-0980 or T-DM1 plus GDC-0980 abrogated PI3K and mTOR signaling molecules (**Figure 2Bi-iii** lane 5 and 6). Still, this inhibition was more with a combination of GDC-0980 with T-DM1 in all cell lines (**Figure 2Bi-iii** lane 6).

Three landmark clinical studies had demonstrated that HER2-positive breast cancer patients with *PIK3CA* mutations are less likely to achieve a pCR compared with those with wild-type, when the patients were treated with neoadjuvant chemotherapy plus anti-HER2 therapies in the GeparQuattro, GeparQuinto, and GeparSixto trials [25], as well as NeoALTO trial [68]. Similarly, PIK3CA mutation is associated with shorter progression-free survival in HER2-positive metastatic breast cancer treated with dual HER2-targeted therapies in the CLEOPATRA trial [69]. However, biomarker data from the EMILIA trial (T-DM1 was used in HER2+ metastatic breast cancer) showed that PIK3CA mutation has hardly any effect on T-DM1 efficacy (PFS: 10.9 months in PIK3CA mutated patients vs. 9.8 months in PIK3CA wild-type patients) [70]. Likewise, T-DM1 was also associated with more prolonged median progression-free survival and overall survival compared to lapatinib plus capecitabine regardless of tumor PTEN expression [70]. However, a phase III MARIANNE data showed that a combination of T-DM1 plus pertuzumab was neither non-inferior nor superior in its efficacy and better tolerability than did taxane plus trastuzumab for first-line treatment of HER2-positive, advanced breast cancer (median PFS: 13.7 months with trastuzumab plus taxane, 14.1 months with T-DM1, and 15.2 months with T-DM1 plus pertuzumab) [71]. More recently, it

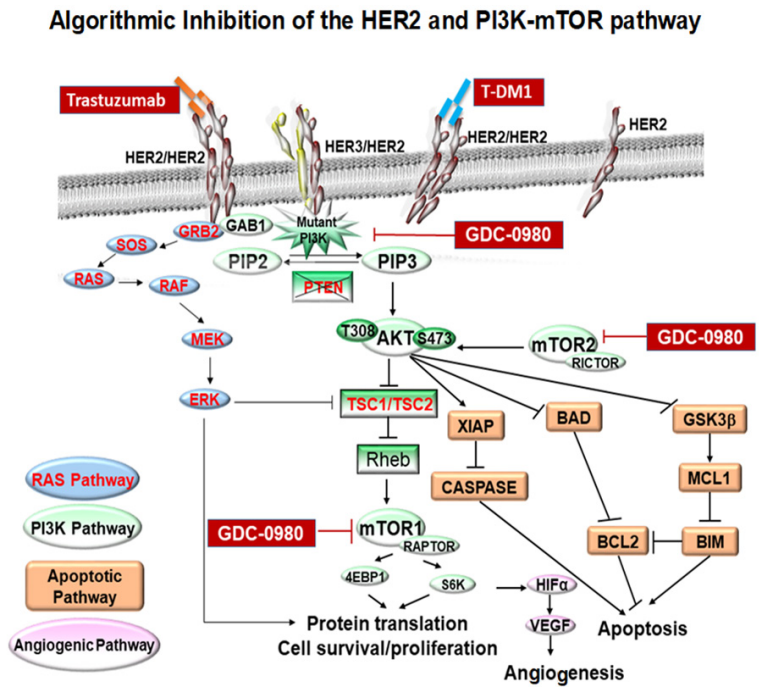

Figure 6. Homo or hetero dimerization of HER2 and HER3 (HER2:HER2 or HER2:HER3) and its downstream RAS-RAF-MEK or PI3K-AKT-mTOR signaling pathway: Activated AKT following stimulation of PI3K pathway drives mTOR-mediated CAP-dependent mRNA translation and also downregulates apoptosis via GSK3beta-BIM, XIAP-caspase and BAD-BCL2 pathways. mTOR regulates HIF1alpha translation, which eventually controls HIF1alpha-VEGF mediated tumor angiogenesis. RAS-RAF-MEK-ERK pathway regulates key signaling for tumorigenesis via protein translation, and cell survival/proliferation. Various targeted inhibitors of HER2 and PI3K-mTORC1/2 such as trastuzumab/T-DM1 and GDC-0980 respectively are presented.

has been reported by several groups that patients with HER2+ metastatic breast cancer who exposed to pertuzumab treatment before T-DM1 have fewer benefits from T-DM1 [72-75]. One of the resistance mechanisms of T-DM1 is the activation of the PI3K pathways by neuregulin (ligand of HER3), which can trigger the formation of HER2: HER3 heterodimerization [4, 76]. It has been identified that the HER2: HERB3 heterodimers are powerful oncogenic units. The phospho-HER3 augments signaling through the PI3K-AKT-mTOR pathway while the p85 regulatory subunit of PI3K initiates downstream signaling via its interaction with phospho-HER3 at 6 consensus PY p85-binding motifs (YXXM).

It has been established that spontaneous formation of HER2: HER3 heterodimer can occur in tumors where HER2 expression on the cell surface is dramatically increased as a consequence of gene amplification. Therefore, it is contemplated that HER3 functions primarily to drive HER2-mediated PI3K signaling, which is essential for tumor cell survival, proliferation, invasion, migration, angiogenesis, and cellular metabolism [7]. From the MARIANNE trial and these above-mentioned studies, it has been realized that HER2: HER3 heterodimerization is an essential factor for HER2 amplified tumor progression, and pertuzumab failed to synergize with T-DM1. Keeping in mind, we sought to define a combination strategy of T-DM1 plus GDC-0980 to suppress HER2: HER3 mediated PI3K-AKT-mTOR signaling.

In all three xenograft models (BT474, BT474HerR, and HCC-1954), anti-tumor responses to single-agent GDC-0980 were observed; however, a combination of GDC-0980 plus trastuzumab or GDC-0980 plus T-DM1 showed more anti-tumor efficacy ranging from tumor growth inhibition to stasis to tumor regression (**Figure 5**). Unlike Violeta Serra and group (using *PIK3CA* mutant overexpressing cells) [77], in our hands, PIK3CA mutant tumor (HCC1954) did not show better response following the single-agent GDC-0980 compared to the other two xenograft models. It was previously reported by others that MYC, a commonly deregulated breast cancer oncogene was responsible for the acquired dual PI3K/mTOR inhibitor resistance [78], and HCC1954 are also MYC-dependent cells [79]. HCC1954 cells have a high copy number of MYC (greater than 7). Additionally, a very high level of amphiregulin (ligand for EGFR) mRNA is also expressed in HCC1954 cells [80]. It has also been reported that amphiregulin-induced EGFR mediated breast cancer cell proliferation is independent of PI3K inhibition [81]. Most importantly, the combination of T-DM1 plus GDC-0980 is the most effective in all three models. Our PD data also demonstrated that both combinations significantly blocked cell signaling (pS6RP), cell proliferation (Ki67), and angiogenesis (CD31

and p-VEGFR2) (**Figure 5B**, **5D**, and **5F**), all are responsible for tumor progression. Our study has several limitations; 1) we have used a limited number of cell lines, 2) we did not include a metastatic brain model, 3) in our HER2 amplified/ER-positive model, we did not use any anti-estrogen in our combination arms and 4) we have not used T-DM1 in lapatinib resistant model.

In summary, a combination of trastuzumab plus GDC-0980 or T-DM1 plus GDC-0980 inhibited the progression of HER2-positive tumors (**Figure 6**). The combination of T-DM1 plus GDC-0890 was found superior to all three models as compared to a single agent or a combination of trastuzumab plus GDC-0980. The result can be explained by our effort to successfully target the survival/proliferation component of the entire "oncogenic event" contributed to HER2 and PI3K-mTOR signals rather than a single gene (HER2)-mediated signals in HER2+ breast cancers. More importantly, in a situation where pertuzumab is not a suitable combination partner with T-DM1 (phase III MARIANNE trial), our data prove that a combination T-DM1 plus GDC-0980 can provide an alternate route of trial design. Here, we measured the signaling impact of GDC0980 alone or in combination with T or T-DM1 *in vitro* and *in vivo*. Interestingly, we investigated whether any relationship exists between anti-HER2 therapy and PD-L1 expression. Our data showed that both anti-HER2 agents significantly induced PD-L1 expression in tumor cells under hypoxic conditions (**Figure 3**). Noticeably, the Neosphere data showed that high PD-L1 expression emerged for its consistent association with lower pCR [82], and it has been recently shown that HER2 signaling regulates the tumor immune microenvironment and trastuzumab efficacy [83]. Although our use of immunocompromised mice was unsuitable for assessing the immune effects of a combination of trastuzumab/T-DM1 plus GDC-0980, the data may provide a rationale to test the role of immunotherapy in the combination of anti-HER2 therapy.

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Disclosure of conflict of interest

None.

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