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Dual mTOR/HDAC Inhibition: Preclinical Development of a Novel Breast Cancer Therapy

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By
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CERTIFICATION OF APPROVAL

I certify that I have read *Dual mTOR/HDAC inhibition: preclinical development of a novel breast cancer therapy* by Mariya Yevtushenko, and I approved this thesis to be submitted in partial fulfillment of the requirements for the degree: Master of Sciences in Biology at Dominican University of California and the Buck Institute for Research on Aging.

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ABSTRACT

HER2 (human epidermal growth factor receptor-2) and/or estrogen receptor (ER) are overexpressed in ~80% of human breast cancers. Although modern therapeutics (e.g. Trastuzumab, Tamoxifen) target the HER2 and ER receptors, clinical resistance often develops due to activation of downstream signaling pathways, including phosphoinositide 3 kinase-AKT/protein kinase B-mammalian target of rapamycin (PI3K/AKT/mTOR), despite effective upstream receptor inhibition. Activation of this pathway increases ribosome biogenesis and translation of oncogenic mRNAs, which are controlled by intracellular histone deacetylase (HDAC) activity as well. In fact, HDAC inhibitors that have been implemented into clinical practice are capable of overcoming resistance to HER2 and ER targeted therapeutics, in part by inducing the degradation of oncogenic transcripts. Given this rationale, we tested the hypothesis that mTOR and HDAC inhibitors are more effective in combination than as single agents. This project examines the effects of combining a novel investigational inhibitor of both complexes MLN0128 that targets mTORC1 and mTORC2, and Trichostatin-A (TSA), a potent inhibitor of both class I and class II HDACs, on the viability, downstream signaling, and polysome assembly of human breast cancer cell lines of various receptor subtypes (HER2-/+ and/or ER -/+), as well as on non-transformed breast epithelial cells. Our data suggest that combining MLN0128 and TSA caused synergistic growth inhibition in almost all breast cancer cell lines tested. Furthermore, this dual treatment induced greater apoptosis in comparison to the single agent treatments in HER2-positive SKBR3 cells, while the viability

and apoptosis of non-transformed MCF-10A cells were less affected. Dual MLN0128/TSA treatment also decreased AKT-S473 phosphorylation significantly more than the single agent treatments in all breast cancer cells, and reduced polysome formation in SKBR3 cells, suggesting that the mechanism of action of dual MLN0128/TSA treatment involves, in part, the inhibition of ribosome function through two separate modes of attack on PI3K/AKT/mTOR signaling. In summary, the synergistic effects of this treatment combination across phenotypically diverse breast cancer cell lines warrants further study and clinical development of this promising breast cancer treatment strategy.

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INTRODUCTION

Clinical subtypes in breast cancer

Breast cancer is one of the highest leading causes of cancer death in the United States. About 12% of women will develop breast cancer over the course of their lifetime [1]. It is a heterogeneous disease with variety of subtypes that are classified by different histologies, levels of gene expression, disease aggressiveness, clinical outcomes, as well as genomic abnormalities [2]. Breast cancer can be classified into three clinical subtypes: estrogen receptor (ER+) or progesterone receptor (PR+) positive, human epidermal growth factor receptor 2 positive (HER2+), and triple negative (ER-/PR-/HER2-) disease [2]. ER+ and PR+ breast cancer overexpresses either the estrogen or progesterone receptors, and is found in 60-80% of breast cancer cases [3]. This type of cancer is more common in older patients, and generally has a better prognosis compared with the other subtypes. In addition, the ER+/PR+ subtype is associated with more differentiated and lower histological grade tumors, which may also play a significant role in the prognostic outcomes since lower grade tumors tend to grow and spread slower [4]. Furthermore, anti-endocrine therapies have been shown to be effective in treating ER+/PR+ breast cancer. For example, the drug tamoxifen– clinically used worldwide for more than 3 decades– is effective in treating many hormone receptor positive cancers because it is an anti-estrogen that blocks the binding of estrogen to ER [5, 6].

HER2+ breast cancers are found in about 20% of patients and are associated with higher mortality rate, along with earlier disease relapse [3]. This

more aggressive clinical subtype tends to have a less positive prognosis, partly due to the elevated proliferative and metastatic potential induced by HER2 [7]. This has led to the development of drugs that specifically target the HER2 receptor, and aid in the treatment of HER2+ breast cancers. The FDA approved Trastuzumab in 1998, which led to the routine use of a clinical assay for HER2 positivity on all breast cancer cases [3]. Trastuzumab is a monoclonal antibody that binds the HER2 receptor and blocks its activation and downstream signaling to inhibit HER2-positive breast cancer cell growth and survival [1]. Some HER2+ breast cancers acquire resistance to Trastuzumab, which has led to the development of multiple approaches to targeting HER2. For example, in 2007 the FDA approved the use of Lapatinib [8], a small molecule inhibitor of the kinase domain of HER2 (and the related epidermal growth factor receptor (EGFR)/HER1 receptor); and in 2012 another HER2 binding antibody, Pertuzumab [9] was also FDA approved for the treatment of HER2-positive breast cancers.

The ER-/PR-/HER2-, or triple negative subtype is associated with aggressive tumors, and this subtype occurs in about 15% of all breast cancer cases [3]. Triple negative breast cancer cells do not express any of the hormone receptors (ER, PR) nor HER2, which prevents their treatment by either ER or HER2 targeted therapies, leaving systemic chemotherapy as the only treatment option [7]. The DNA damage response mechanisms play important roles in many triple negative breast cancers, as the tumor suppressors p53, BRCA1, and BRCA2 are often mutated in this subtype. Research has shown that p53 is

mutated in about 82% of triple negative cancers [3]. Additionally, BRCA1 or BRCA2 mutations occur in up to 10% of triple negative cancers, and women with either mutation are at a 45-75% lifetime risk of developing breast cancer in their lifetimes [10]. Increased mitogenic signaling may also drive the proliferation of some triple negative breast cancers. Recent studies carried out by the Cancer Genome Atlas indicate that PIC3CA, a gene encoding for an isoform of phosphoinositide 3 kinase (PI3K), is mutated in about 9% of triple negative breast cancers [11]. The triple negative subtype is also more common in younger patients, and in women of African descent. Tumors in these patients are often found poorly differentiated and higher grade, which further contributes to their poor prognosis [4].

HER2 and the PI3Kinase/Akt/mTOR signaling pathways in breast cancer

HER2, also known as ErbB2/*neu*, is a receptor tyrosine kinase (RTK), and is one of the four members of the epidermal-like growth factor family of receptors (EGFR). The other receptors of the EGFR family are EGFR/HER1, HER3, and HER4 [12]. All four of the transmembrane receptors can form homodimers and heterodimers, which can be enhanced by ligand binding. HER2 is referred to as an “orphan receptor” because unlike the other three it does not directly or singly interact with any known ligand. However, it dimerizes with other ligand-binding EGFR family members to modulate HER2 tyrosine kinase activity negatively or positively depending on the ligand/dimerization partner [13]. Upon dimerization, the HER2 receptor undergoes auto-or-trans-phosphorylation. As mentioned

previously, the overexpressed and phosphorylated HER2 receptor activates the downstream phosphoinositide 3 kinase, protein kinase B (also known as AKT), mammalian target of rapamycin (PI3K/AKT/mTOR) pathways, which increase cell proliferation and survival [14] (Figure 1).

PI3Ks are part of a family of lipid kinases that phosphorylate phosphatidylinositol at the 3' hydroxyl group [15, 16]. The dysregulation of the PI3K pathway has been found in a variety of cancers [16]. The catalytic subunit of PI3K, p110α is involved in regulating cell proliferation, and plays an important role in tumorigenesis [17]. Gain-of-function mutations in p110α, specifically in its helical and kinase domains, are found in about 30% of tumors, including breast, and are associated with increased AKT signaling [17]. Wild type PI3K can be activated by RTKs, via adaptor proteins that sequester to its inhibitory subunit p85, thereby activating the p110 kinase domain [16].

Activated p110 phosphorylates PIP_2, (phosphatidylinositol 4,5-biphosphate) thus producing PIP_3 (phophatidylnositol-3, 4,5 triphosphate), a lipid second messenger [18]. Conversely, PIP3 is negatively regulated via dephosphorylation by PTEN (phosphatase and tensin homolog) a tumor suppressor protein; however in many cancers, PTEN is inactivated or lost leading to increased levels of PIP_3 [16]. $PIP_{3,}$ subsequently binds to PDK1 (3-phosphoinositide dependent protein kinase-1), which phosphorylates and activates AKT [16, 19-21].

AKT is a serine/threonine kinase that controls protein synthesis and cell growth by phosphorylating mTOR (mammalian target of rapamycin) [19]. AKT

has three isoforms, AKT 1/2/3, as well as at least two phosphorylation sites, S473 and T308. Phosphorylation on both sites increases the kinase activity and causes AKT to dissociate from the membrane, allowing it to activate the TORC1 complex and other downstream substrates [18].

mTOR is a serine/threonine kinase that regulates cell growth, proliferation, survival, and metabolism [22]. mTOR has two multi-protein complexes, mTORC1, and mTORC2. mTORC1 consists of mTOR, mLST8 (G-protein β-subunit-like protein) and raptor (regulatory associated protein of mTOR). This complex is involved in regulating cell growth, proliferation, survival, as well as nutrient and energy signals [23]. Furthermore, mTORC1 has been shown to phosphorylate p70 S6 kinase (S6K1), and eukaryotic initiation factor 4E- (eIF4E) binding protein 1 (4eBP1) [23]. In comparison, mTORC2 consists of mTOR, mLST8, and rictor (rapamycin-insensitive companion of mTOR). Unlike mTORC1, mTORC2 has been shown to regulate the actin cytoskeleton, and phosphorylates AKT on the S473 residue to modulate cell shape, motility, and invasive abilities [23].

In mammals there are two ribosomal S6 kinases, S6K1 and S6K2, whose activation is regulated by mTOR [23-25]. A substrate of S6 kinases is the ribosomal protein S6, which is also referred to as RPS6. S6 controls cell growth by affecting mRNA translation and protein synthesis. mTOR activated S6K1 promotes translation and protein synthesis by phosphorylating the 40S ribosomal protein S6, as well as the translation initiation factor eIF4B [23].

4eBP1, is another downstream target of mTOR, that acts as a repressor of eIF4E [23]. When 4eBP1 is hypo-phosphorylated, it binds to eIF4E and represses cap-dependent translation to prevent eIF4E from binding to eIF4G. However, when 4eBP1 is phosphorylated by mTOR, it dissociates from eIF4E, which allows for different initiation factors including the 40S ribosomal subunit to be positioned at the 5' end of the mRNA to help start the scanning process of the mRNA [23]. Elevated levels of eIF4E have been found in a variety of cancers, including breast cancer [26]. Furthermore, overexpression of eIF4E has been shown to be tumorigenic by dysregulating translation of oncogenes [24]. Given this information, researchers have greatly focused on mTOR inhibition in order to identify new and more effective anticancer agents. mTOR inhibitors such as rapamycin-related rapalogs (e.g. FDA approved Everolimus and Temsirolimus), that inhibit TORC1, have been shown to reduce tumor angiogenesis, and have clinical anti-tumor activity [22]. However, they are unable to inhibit TORC2. Studies have shown that inhibition of TORC1 alone up-regulates AKT phosphorylation which can lead to tumor progression. Specifically, activated S6K phosphorylates, and negatively regulates, insulin receptor substrate 1 (IRS-1), ultimately leading to decreased PI3K and AKT activity. Consequently, inhibition of TORC1 causes reduced S6K activity, and an increase AKT. However, inhibition of TORC2 decreases AKT activity. Thus, inhibiting both mTOR complexes could prevent activation of that feedback loop and result in improved therapeutic effects [23].

Mammalian target of rapamycin inhibitors

Intellikine Inc. (recently acquired by Takeda/Millennium Pharmaceuticals) developed the selective TORC1/2 ATP site inhibitor MLN0128 (previously known as INK128). Unlike rapamycin and rapalogs that allosterically inhibit TORC1 and only weakly inhibit p4eBP1 levels, MLN0128 is able to decrease the phosphorylation of S6, and inhibit p4eBP1 levels [27-29]. MLN0128 also decreases AKT activity since it inhibits TORC2 as well (Figure 1). MLN0128 has been shown to inhibit the proliferation of various subtypes of breast cancer cell lines with high potency and to potently inhibit growth in human breast cancer xenograft models [29]. Additional research demonstrated that MLN0128 can suppress the progression of leukemia, and may have therapeutic effects in treating B-cell acute lymphoblastic leukemia [30].

Histone Deacetylases (HDACs) and their inhibition

Aside from the mutations in the PI3K/AKT/mTOR pathway, there are epigenetic mechanisms that commonly contribute to the development of breast and other cancers. Posttranslational modifications such as acetylation and methylation affect gene expression, and may contribute to cancer development [31]. Histone deacetylases (HDACs) remove acetyl groups from histones and various other non-histone proteins to modulate gene expression [31, 32]. For genes like the tumor suppressor p21, an increase in HDAC activity can repress its transcription. High expression of HDACs has been documented in many different cancers implicating its importance in the carcinogenesis process [32].

Furthermore, a global pattern of histone hypoacetylation has been associated with malignant progression of breast cancer [33]. There are three classes of mammalian HDACs. Class I includes HDAC1, HDAC2, HDAC3, and HDAC8. This class of HDACs are mainly localized in the nucleus [34]. Class II consists of HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10. This class of HDACs can move between the nucleus and the cytoplasm [34]. Class III HDACs– structurally unrelated to the class I or II HDACs– are composed of a family of NAD-dependent sirtuins (SIRs). The SIRs are unaffected by all of the class I or II HDAC inhibitors developed to date [34]. It is also interesting to note that higher expressions of HDAC4, HDAC8, and HDAC9 have been found in tumor tissues in comparison to normal tissues [34].

Better understanding of HDACs and their role in tumorigenesis has fueled the development of HDAC inhibitors (HDACi's) as a new type of cancer therapeutic [34]. HDAC inhibitors prevent HDAC's from removing acetyl groups from histones and non-histone proteins and, therefore, can alter gene expression and reverse the diverse tumorigenic effects of increased HDAC activity [34]. While HDAC inhibitors have been shown to inhibit angiogenesis and metastasis in pre-clinical models, less is known about the mechanism of how they kill tumor cells while sparing normal cells [31].

The currently available HDACi's– referred to as pan-HDACi– inhibit both class I and class II HDACs. In 2006, the first pan-HDACi inhibitor, Vorinostat was clinically approved for the treatment of cutaneous T cell lymphoma. In 2009, the FDA approved another such pan-HDACi, Romidepsin, for the same clinical

indication [31]. Panobinostat and Entinostat are two newer HDACi's whose clinical development includes trials in patients with advanced stage breast cancer [31, 35].

In addition to pan-HDACi, class-specific HDACi are also in preclinical and clinical development. For example, recent studies have shown that Entinostat, a class I HDAC inhibitor, is able to induce apoptosis in breast cancer cells, as well as down regulate HER2 and HER3 [35]. In addition, class II specific HDACi's such as MC1568 and MC1575 show anti-proliferative activity in human melanoma cells [36], as well as in human breast cancer cells [37].

This study focuses on a potent pan-HDAC inhibitor, Trichostatin-A (TSA), an antifungal antibiotic that acts as a reversible non-competitive inhibitor [38]. TSA remains in preclinical studies and has been shown to promote cell cycle arrest in the G_1 and G_2 phases, and in some cases promote apoptosis. In some cancer cells, TSA has been shown to induce differentiation rather than promote cell death [38]. TSA (and other pan-HDACi's) has also been shown to induce HER2 mRNA degradation [39]. Furthermore, unpublished data from the Benz lab have shown that the addition of cyclohexamide to HER2 positive SKBR3 breast cancer cells inhibits polyribosome (polysome) formation and reverses the effects of TSA, suggesting that polysome function and active translation appear to be necessary for HDACi to promote HER2 mRNA degradation.

Research Goals

The focus of this project was to determine the therapeutic potential and anti-tumor mechanism of action of mTOR and HDAC inhibitors, represented by MLN0128 and TSA respectively, as individual anticancer agents and in therapeutic combination, and to determine if their potential utility is dependent on a specific breast cancer phenotype, be it hormone receptor (ER, PR)-positive, HER2-positive, or neither. Since breast cancer cell lines modeling these different breast cancer clinical phenotypes are readily available, we examined the effects of these inhibitors on breast cancer cell viability and determined how these phenotypically different model breast lines respond to MLN0128/TSA treatment given as single agents or in combination. To understand the antitumor mechanism(s) of these drugs with regard to phosphorylation events in the PI3K/AKT/mTOR signaling pathway, we determined if adding TSA to MLN0128 impairs or enhances activation of AKT, S6, and/or 4eBP1. Since unpublished data from the Benz lab also indicated that polysome function is necessary for TSA to induce HER2 mRNA decay, we further evaluated the possibility that mTOR inhibition could antagonize the anti-tumor effects of TSA by inhibiting polysome function. Furthermore, since HDACi's can impair cancer cell growth, we determined whether its growth inhibiting interaction with MLN0128 induces apoptosis in breast cancer cells relative to non-transformed MCF-10A cells.

MATERIALS AND METHODS

Cell Culture

Human breast cancer cells lines expressing different levels of ER/PR and HER2 levels were cultured under sterile conditions and incubated at 37°C in 5% CO_2. SKBR3 cells (HER2+/ER-) were grown in McCoy's medium and supplemented with 10% Fetal Bovine Serum (FBS). MDA-MB-231 (ER-/PR-/HER2-), or triple negative cell line, and JIMT-1 (HER2+/ER-/PR-) Herceptin resistant cells, were grown in DMEM and supplemented with 10% FBS. MCF7 (ER+/PR+/HER2-) cells, and MCF7 HER2-18 (ER+/PR+/HER2+) cell line stably overexpressing HER2 45x endogenous levels as described [41] were grown in DMEM and supplemented with 10% FBS and 1% insulin. MCF-10A (ER-/PR-/HER2-) immortalized but non-tumorigenic mammary epithelial cell line was grown in 50% DMEM and 50% Ham's F12 media supplemented with 5% horse serum, 2mmol/L L-glutamine, 0.02 mmol/L non-essential amino acids, 10ng/ml EGF, 0.5µg/ml hydrocortisone, 0.1µg/ml cholera toxin, and 10µg/ml of insulin. BT-474 (ER+/PR+/HER2+) cell line was grown in RPMI 1640 medium, and supplemented with 10% FBS. The JIMT-1 cells were obtained from AddexBio (San Diego, CA, USA) all of the other cell lines were originally obtained from the (ATCC) American Type Culture Collection (Manassas, VA, USA).

Drugs

MLN0128 (INK128) was obtained under a Material Transfer Agreement from Intellikine, Inc. (Millennium Pharmaceuticals, Cambridge, MA, USA). TSA

and MC1568 were obtained from Sigma Aldrich (St. Louis, MO, USA). Entinostat was obtained from Syndax Pharmaceuticals Inc. (Waltham, MA, USA).

Cell Viability Assay

CellTiter-Glo Cell Viability Assay (Promega, Madison, WI, USA) was used to determine cell viability after single agent and combination treatments with TSA, MLN0128, Entinostat and MC1568 according to manufacturer's protocol. SKBR3 and BT-474 breast cancer cell lines were plated at 5000 cells/well, and MCF7 NEO3, MCF7 HER2-18, MCF-10A, MDA-MB-231, and JIMT-1 cells were plated at 2500 cells/well in a 96 well plate. (Equal confluences of each cell line were obtained). After a 24-hour period where the cells were allowed to attach, the cells were treated with DMSO (as a positive control) 0nM, 25nM, 50nM, and 100nM concentration of TSA and 0nM, 0.2nM, 1nM, 5nM, and 25nM concentration of MLN0128 for 72 hours. Control cells were treated by vehicle used to dissolve each drug. To determine the growth inhibitory effects of TSA, Entinostat, and MC1568, SKBR3 cells were plated in a 96 well plate at 5000 cells/well. Cells were allowed to attach for 24 hours and then treated with DMSO, 0nM, 125nM, 250nM, 500nM, and 1000nM of each drug for 72 hours. Cell viability was quantified using a Fluoroskan luminometer (Ascent, FL). IC_{50} values for MLN0128 and TSA were calculated using GraphPad Prism version 5.0. Synergism interactions were analyzed using Calcusyn Software (Biosoft, Great Shelford Cambridge, UK).

Western Blot Analysis

SKBR3 and BT-474 cells were plated at approximately 5.5 x 10^5 cells/well, and MCF7 NEO3, MCF7 HER2-18, MCF-10A, and JIMT-1 cells were plated at approximately 2.5 x 10^5 cells/well in a 6-well plate. The cells were allowed to attach for 24 hours, and were treated with the DMSO, 0nM, 25nM, 50nM, and 100nM concentration of TSA and 0nM, 0.2nM, 1nM, 5nM, and 25nM concentration of MLN0128 for 8 hours. Whole cell lysates were washed with Phosphate-Buffered Saline (PBS) and harvested in RIPA buffer (10 mM Tris-CL (pH 8.0), 1mM EDTA, 0.5 mM EGTA, 1% triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl) containing complete Mini, EDTA-free Protease Inhibitor Cocktail and PhosSTOP Phosphatase Inhibitor Cocktail tablets, (Roche, Indianapolis, IN, USA). Cell lysates were then centrifuged at 14,000rpm, pellets discarded, and supernatants were stored in -80°C.

Protein concentration was determined using the Bradford Coomassie Assay (BCA) kit (Pierce, Rockford, IL, USA). Protein concentrations among the samples were normalized by the addition of appropriate amounts of RIPA buffer, After the addition of 2X SDS sample buffer, samples were separated by SDS-PAGE gel electrophoresis using NuPAGE 4-12% Bis-Tris-Gel (Life Technologies, Grand Island, NY, USA). Proteins were transferred onto a PVDF membrane (COMPANY), and membrane was blocked for 1 hour in 4% nonfat dried milk in 1X TBST (Tris buffered saline with 0.05% Tween 20 pH 8.0 (Sigma Aldrich, St. Louis, MO, USA)). Western blot analysis was performed using protein-specific antibodies: pS6 S240/244 rabbit monoclonal antibody (mAb), p4eBp1 S65 rabbit

mAb, 4eBp1 rabbit mAb, pAKT-S473 rabbit mAb, AKT rabbit polyclonal antibody, Beta-tubulin rabbit polyclonal antibody, pS6 rabbit mAb, S6 rabbit mAb, and Cleaved PARP rabbit mAb (Cell Signaling, Danvers, MA, USA). Mouse monoclonal HER2 antibody was obtained from Cal Biochem (now Millipore, Billerica, MA, USA) and rabbit polyclonal pHER2 antibody was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The antibodies were detected using secondary antibodies conjugated to horse radish peroxidase and luminol as a substrate (Thermo Fisher Scientific, Rockford, IL, USA).

Polysome Fractionation

SKBR3 cells were plated at approximately 1.2 x 10^7 cells per 15cm plate, and were allowed to attach for 24 hours. Cells were treated with the DMSO (as a positive control), 0nM, and 50nM of MLN0128, and 0nM, and 500nM concentrations of TSA, in single agent and combination for 24 hours. Cells were then treated with 50µg/ml cyclohexamide for 15min to immobilize ribosome complexes. After removing media and washing harvested cells with (Dulbecco's Phosphate-Buffered Saline (DPBS), SKBR3 cells were scraped and transferred to dounce homogenizer on ice, and homogenized with lysis buffer (0.1M sodium orthovanadate, protease inhibitor, phosphatase inhibitor, 50µg/ml cyclohexamide, RNAse inhibitor, and NP-40 buffer). The samples were spun at 14,000rpm for 5 minutes, and pellet was discarded. The samples were snap frozen in liquid nitrogen, and stored in the -80°C until day of polysome fractionation.

Polysome profile analysis was employed as recently described [40]. A sucrose gradient was poured by layering 10%-50% sucrose buffers. Gradients were kept at room temperature for 1hr and 45 minutes to form continuous gradient, and placed in the -80°C until the day of polysome fractionation. On the day of fractionation, the samples were layered on top of the continuous sucrose gradient and the samples were spun at 38,000 rpm for 2 hours at 4°C. The gradients were fractioned and polysome fractions were then collected by utilizing the Retriever 500 with ISCO Teledyne UV/Vis (UA6).

Apoptosis Assay

SKBR3 cells were plated at approximately 4 $x10^5$ cells/well in a 6 well plate. Twenty-four hours later, cells were treated with 0nM and 100nM concentration of TSA, and 0nM and 25nM concentration of MLN0128. SKBR3 cells treated with 5µg/mL Adriamycin served as a positive control for apoptotic cells. Whole cells lysates were prepared by the same methods stated above and Western blot analysis was used to analyze PARP cleavage using anti-PARP (46D11) rabbit monoclonal antibody obtained from Cell Signaling (Danvers, MA, USA).

RESULTS

Combination of TORC1/2 and pan-HDAC inhibitors synergistically decreases breast cancer cell viability

To assess the effect of single agent and combination treatment using the TORC1/2 inhibitor, MLN0128, and the pan-HDACi TSA, we utilized the CellTiter-Glo assay to measure cell viability in a variety of breast cancer cells (Figure 2). IC_{50} values for MLN0128 and TSA ranged from 4.64nM to 25nM, and 30.9nM to100nM, respectively (Figure 3). Among all the cell lines tested, SKBR3 cells appeared to be the most sensitive to single agent and dual treatment with both drugs (Figure 2A). The IC_{50} for MLN0128 in SKBR3 cells was 4.64nM, and 30.9nM for TSA (Figure 3). MCF7 NEO3 cells were also sensitive to treatment with MLN0128 and TSA, with IC_{50} values of 14.1nM for MNLO128, and 63.1nM for TSA (Figure 2B, and 3). The most resistant cell lines were the MCF7 HER2-18, BT-474, JIMT-1, and MCF-10A (Figure 2C, 2D, 2E, 2G). These cell lines had IC_{50} values ranging from 14.1nM to 25nM for MLN0128, and 63.1nM to 100nM for TSA (Figure 3). MDA-MB-231 cells were more sensitive to MLN0128 with an IC_{50} value of 11.75nM, but less affected by TSA with an IC_{50} value of 89.13nM (Figure 2F and 3). The non-malignant mammary epithelial cells, MCF-10A were the most resistant, and had IC_{50} values of 25.1nM and 100nM for MLN0128 and TSA, respectively (Figure 2G, and 3). There were no correlations observed with regard to known breast cancer phenotype or genotype, including ER and HER2 status or PI3K mutation status (Figure 3).

In order to determine if additive, synergistic, or antagonistic interactions exist between MLN0128 and TSA, we used Calcusyn software to calculate the combination index (CI) (Figure 4). A combination index of 1 indicates that two drugs are acting in an additive manner. CI values less than 1 represent synergism while values greater than 1 represent antagonism. CI was calculated for the cells that were sensitive enough to MLN0128 and TSA and we found that the overall effects of combining MLN0128 and TSA showed synergistic interaction with CI values ranging from 0.264-0.898 (Figure 4). The MDA-MB-231 cells appeared to show an antagonistic effect at low doses of MLN0128 and TSA (25nM and 50nM), however higher doses (25nM and 100nM) of both drugs showed the strongest synergistic interaction, with a CI of 0.264, compared with all other cell lines tested (Figure 4). Furthermore, nearly additive effects were observed in the MCF7 HER2-18 cells, at the 25nM MLN0128 and 50nM TSA dose with a CI value of 0.903 (Figure 4). These results show that combination of MLN0128 and TSA synergistically inhibit breast cancer cell viability compared with single agent treatment using either drug alone.

TORC1/2 inhibitor blocks downstream signaling effectors

To examine the early effects of MLN0128 and TSA on the PI3K/AKT/mTOR pathway, various breast cancer cell lines were treated for 8-hours with concentrations similar to the ones used in the viability assays (Figure 5). MLN0128 alone reduced phosphorylation levels of S6 and 4eBP1 in all cell lines. While single agent treatment of TSA did not have an effect on the

phosphorylation of S6 or 4eBP1, the combination of MLN0128 and TSA showed a reduced phosphorylation of 4eBP1 in SKBR3, MCF7 HER2-18, and MCF-10A cells compared to single agent treatments of either drug (Figure 5A, 5C, and, 5F). As expected, MLN0128 treatment reduced AKT S473 phosphorylation in all cell lines tested and TSA alone reduced AKT phosphorylation in the cell lines most responsive to TSA treatment: SKBR3, MCF7 NEO3, MCF7 HER2-18 (Figure 5A, 5B, 5C). In addition, phosphorylation of AKT on S473 was further reduced with dual MLN0128/TSA treatment as compared to the single agent treatments in all cell lines tested (Figure 5). Even in cell lines where TSA treatment alone did not affect AKT phosphorylation (BT-474, JIMT-1, MCF-10A), adding TSA at 100nM further decrease AKT phosphorylation (Figure 5D, 5E, 5F).

To determine the effects of long-term treatment with MLN0128 and/or TSA on PI3K/mTOR/AKT pathway signaling events, we evaluated 24-hour treatments of both drugs as single agents, or in combination at varying concentrations of TSA (0nM, 50nM, and 1000nM) (Figure 6). Although low doses of MLN0128 decreased the phosphorylation of S6 and 4eBP1, very high doses of TSA (1000nM) were necessary to block S6 and 4eBP1 phosphorylation. Furthermore, high doses of TSA in combination with MLN0128 further inhibited S6 and 4eBP1 phosphorylation when compared to either of the single agent treatments (Figure 6), suggesting that both drugs are capable of modulating S6 and 4eBP1 phosphorylation with MLN0128 being more potent than TSA.

As previously mentioned, unpublished Benz Lab data shows that TSA promotes HER2 mRNA degradation in a polysome dependent manner. Since

MLN0128 affects the phosphorylation of ribosomal proteins, including S6 (Figure 5), we next examined whether MLN0128 can prevent TSA-mediated reduction in HER2 levels. Results in figure 7 indicate that HER2 protein levels were decreased following a 24-hour treatment with 1000nM of TSA even in the presence of MLN0128 (Figure 7). While TSA did not alter HER2 protein levels significantly within 24 hours of treatment, it did reduce pHER2 to a non-detectable level and blocked HER2 activation. MLN0128 did not appear to have any effect on HER2 activation or total HER2 levels in absence of TSA, nor did it appear to interfere with the effects of TSA on HER2 expression or phosphorylation (Figure 7). The observed decrease in HER2 protein levels may be attributed to mRNA degradation; further testing will be necessary to examine the effects of TSA and MLN0128 on HER2 mRNA levels.

Inhibitors of TORC1/2 and HDACs decrease polysome formation

The PI3K/AKT/mTOR pathway is known to affect posttranslational modifications on ribosomal proteins and translation initiation factors [28], Since sustained TSA reduced S6 phosphorylation after 24 hours of treatment (Figure 6) we investigated the effects of MLN0128 and TSA on polysome formation and structure. We used a 50nM concentration of MLN0128 (10x the IC_{50} value) and a 500nM of TSA (16x the IC_{50} value). MLN0128 alone caused a subtle decrease in polysome formation, as did TSA as single agents. The combination of MLN0128 and TSA significantly blocked polysome formation, greater than either of the

drugs alone (Figure 8). These results support the convergent influence of MLN0128 and TSA in decreasing polysome formation, at least in SKBR3 cells.

Combined treatment with TORC1/2 and HDAC inhibitors induces apoptosis in breast cancer cells

MLN0128 and TSA have been shown to lower the viability of breast cancer cells and, and to a lesser extent, non-transformed mammary epithelial cells (Figure 2). We investigated whether this difference in viability is related to increased apoptosis in SKBR3 cells in response to MLN0128 and/or TSA. To accomplish this, we examined PARP cleavage as a marker for apoptosis in SKBR3 (HER2+/ER-) cells and non-transformed MCF-10A mammary epithelial cells. Cells were treated for 48 hours with 25nM of MLN0128 and 100nM of TSA (highest concentrations used in the viability experiments) alone or in combination, with adriamycin treated cells used as a positive control for PARP cleavage (Figure 9). In SKBR3 cells, MLN0128 or TSA alone caused only a small increase in cleaved PARP levels, however, this cleavage was further enhanced when the two drugs were given in combination (Figure 9). No PARP cleavage was observed in the MCF-10A cells, with any of the MLN0128 and/or TSA treatment doses (Figure 9). Taken together, our results show that MLN0128 and TSA alone or in combination inhibit SKBR3 breast cancer cell growth by promoting apoptosis, whereas these drugs appear to have little effect on non-transformed MCF-10A cells.

Class I HDACi showed greater reduction in breast cancer cell viability compared to class II HDACi.

In order to determine whether the growth inhibitory effects of HDAC inhibitors are class I or class II dependent, we treated SKBR3 cells with Entinostat (class I HDACi), and MC1568 (class II HDACi), as well as TSA (pan-HDACi) (Figure 10). Cells were treated with the aforementioned drugs for 72 hours and a cell viability assay was performed as before. Results in figure 10 indicate that TSA is more potent in inhibiting cell viability in comparison to the other two HDACi's. While Entinostat displayed growth inhibitory effects at higher dosages, MC1568 did not appear to have any inhibitory effects, even at the highest concentration of 1000nM (Figure 10). However further research with other class I and class II specific HDACi's are necessary to have a better understanding of these effects.

DISCUSSION

HER2 overexpression and phosphorylation, and the activation of the PI3K/Akt/mTOR pathway have been linked to increased breast cancer cell survival, growth, and metastasis [7, 16, 17]. HER2 amplification has been associated with very poor prognosis in breast cancer patients [7] with a variety of resistance mechanisms developing either de novo or soon after clinical treatment with HER2 targeted therapies. In this study, we tested the therapeutic potential of a potent and investigational mTORC1/2 inhibitor, MLN0128, as a stand-alone treatment and in combination with a pan-HDAC inhibitor, TSA. Studies were conducted to evaluate the effects of these drugs alone or in combination on cell viability, PI3K/Akt/mTOR signaling, and polysome formation.

Our data show that MLN0128 and TSA synergistically inhibit cell viability when administered in combination (Figure 2). While both drugs were effective in blocking cell viability in a dose-dependent manner against a panel of phenotypically diverse breast cancer cell lines, we did not observe any patterns of responsiveness that correlated with a specific breast cancer clinical (i.e. HER2 or ER receptor) phenotype (Figure 3). Interestingly, MLN0128 and TSA as single or combination treatments, appeared to have little effect on non-malignant mammary epithelial cells, MCF-10A (Figure 2G). These results suggest that MLN0128 and TSA given in combination could exhibit antitumor activity *in vivo* without significant toxicity to non-malignant cells; however, this conclusion requires additional preclinical *in vivo* testing.

In addition to inhibiting cell viability, studies have shown that MLN0128 is effective in blocking mTORC1/2 signaling which promotes survival of cancer cells by regulating proliferation, growth, metabolism, invasiveness, and motility [22]. More specifically, MLN0128 has been reported to inhibit pS6, p4eBP1, and pAKT-S473, in preclinical studies [27-30]. In line with these studies, our results also indicate that MLN0128 inhibits phosphorylation of S6 and 4eBP1 (Figure 5), both of which are downstream effector molecules of mTOR [23, 25]. Furthermore, earlier studies have indicated that inhibition of mTORC1 alone may not be sufficient because mTORC1 inhibitors (rapalogs) have been shown to increase the phosphorylation of AKT on S473 [23, 29]. Consistent with this, data from this study also show that MLN0128 is able to inhibit AKT S473 phosphorylation. We also demonstrated that TSA in combination with MLN0128 further reduces the phosphorylation of AKT S473, even though, in most cell lines tested, TSA alone has little if any effect on pAKT-S473 after 8 hours of treatment (Figure 5). Although TSA was not able to inhibit pS6 and p4eBP1 after 8 hours of treatment, at a longer time point and higher doses, TSA did reduce phosphorylation levels of S6 and 4eBP1 when used in combination with MLN0128 (Figure 6). Taken together, these findings suggest that pS6 and p4eBP1 could serve as robust biomarkers for MLN0128 response, and that pAKT-S473 may serve as a response biomarker for the MLN0128 and TSA combination therapy.

Since polysome function contributes to HER2 protein expression and is necessary for TSA to induce HER2 mRNA decay, we examined the effects of

MLN0128 and TSA on polysome formation. Similar to the cell viability results, the combination treatment of MLN0128 and TSA at concentrations above their IC_{50} values was more effective at decreasing polysome formation than the individual drugs alone (Figure 8).

The results of our viability studies indicate that MLN0128 and TSA synergistically inhibit viability of a variety of breast cancer cells lines, and to a lesser degree, non-transformed mammary epithelial cells. We found that, in SKBR3 cells, that reduction in viability is caused, at least in part, by induction of apoptosis; as single agents, both MLN0128 and TSA increased PARP cleavage, while the combination treatment showed an even stronger effect on this apoptotic marker (Figure 9). In contrast, the non-transformed MCF-10A cells did not show any significant induction of apoptosis, which underscores the potential clinical use of these two drugs in combination.

Since TSA is a pan HDACi that targets both class I and class II HDAC's, we investigated whether the reduction in cell viability is dependent on class I HDACs, class II HDAC's, or on both classes. We found that treatment with one specific class I HDACi caused greater reduction in cell growth in comparison with the treatment of a different class II HDACi (Figure 10), implicating the greater contribution of class I HDACs in regulating breast cancer cell growth and potentially accounting for the observed synergy with MLN0128. However, further evaluation with additional class-specific HDACi's is necessary to firmly establish this conclusion.

Further research to better understand the interaction between TORC1/C2 inhibition and HDAC inhibition as a new therapeutic strategy are necessary. It will be interesting to examine if MLN0128 affects canonical TSA-induced events, such as increased p21 levels [31]. More importantly, although we examined class-selective HDACi, it will be critical to determine if the synergistic growth inhibiting effects induced by the combination treatment depend mostly on TSA inhibition of class I, class II or both classes of intracellular HDACs. Also, the finding that MLN0128 and TSA induced-apoptosis is observed in SKBR3 cells, but not in the non-transformed MCF-10A's, leads us to wonder if the drugs can induce senescence in MCF10A cells. Such additional studies will enhance the preclinical rationale to warrant further development of targeted therapy focused on mTORC1/C2 and HDAC.

CONCLUSION

Our data indicate that the combination of an mTORC1/C2 inhibitor like MLN0128 and a pan-HDACi like TSA is more effective at inhibiting cell viability, polysome function, and AKT phosphorylation, and inducing apoptosis, than single agent treatment by either drug alone. In addition to the aforementioned convergent mechanisms of action, both MLN0128 and TSA display drug-specific molecular events; for example, MLN0128 is more robust in reducing S6 and 4eBP1 phosphorylation, while TSA, but not MLN0128, reduces HER2 activation and receptor levels. Taken together, the results from this study demonstrate that both drugs inhibit cell growth and promote apoptosis in various breast cancer cell lines through both convergent (i.e. both drugs decrease AKT phosphorylation) and divergent pathways (MLN0128 is a much more potent inhibitor of S6 and 4eBP1 phosphorylation compared to TSA). Further research with this innovative therapeutic drug combination may lead to the development of more effective anticancer treatment regimens, especially for HER2-positive breast cancers resistant to current anti-HER2 therapeutics.

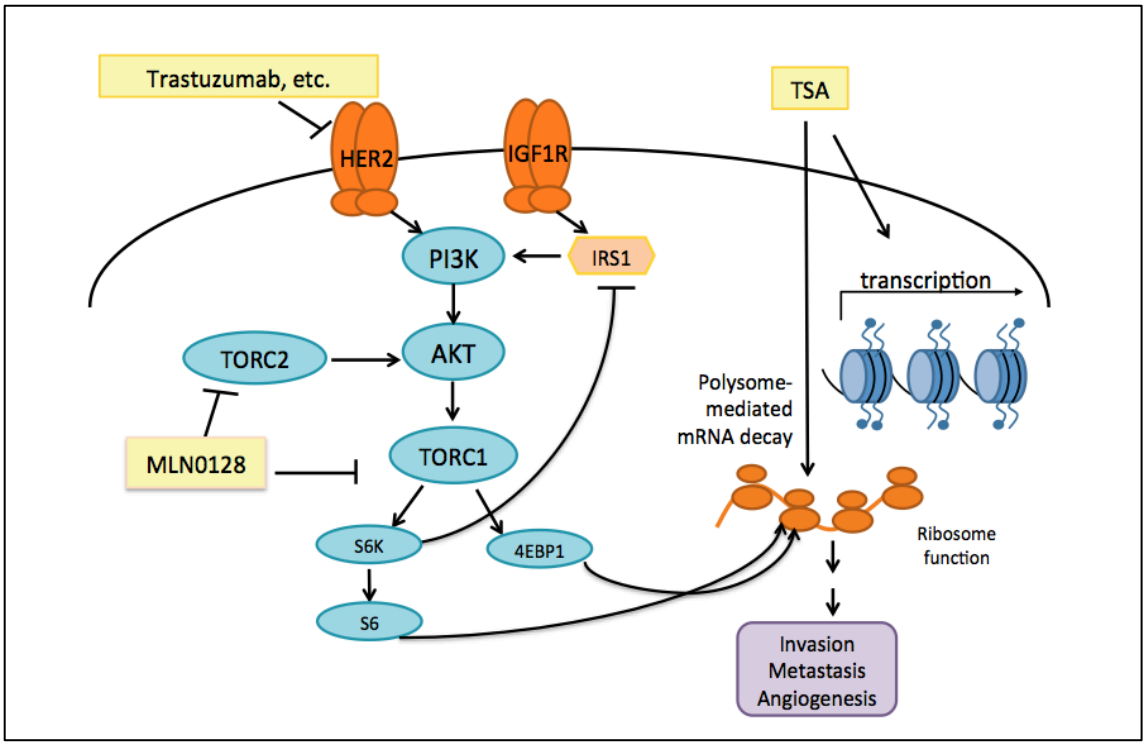
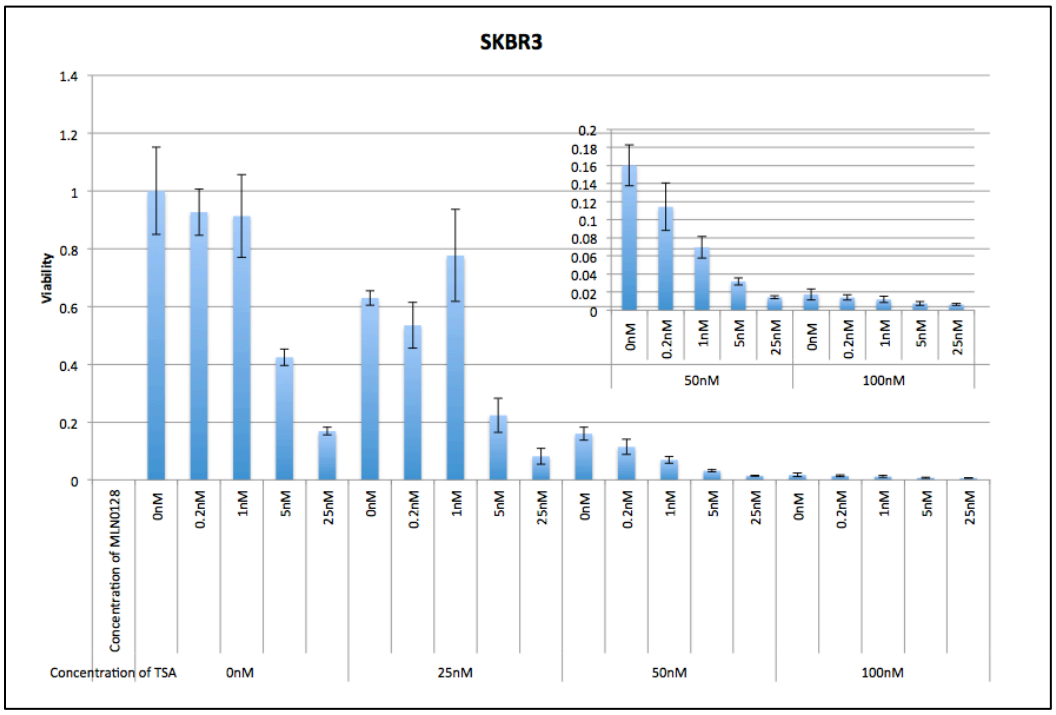


Figure 1. Schematic of signaling pathways targeted by MLN0128 and TSA. HER2 (and other RTK's) dimerization and auto-phosphorylation leads to PI3K/AKT/mTOR activation and subsequently the phosphorylation of downstream targets of mTOR. Phosphorylation of S6K and 4eBP1 results in increased cell proliferation and metastasis. mTOR inhibitor MLN0128 (INK128) blocks both TORC1 and TORC2 to inhibit the functions of both S6K and 4eBP1. Inhibition of TORC2 by MLN0128 decreases Akt activity and prevents IRS-1 from further up-regulating AKT after mTORC1 inhibition [42]. The HDACi TSA mediates HER2 mRNA decay through a polyribosome-dependent mechanism.

A


SKBR3
Viability
Concentration of MLN0128
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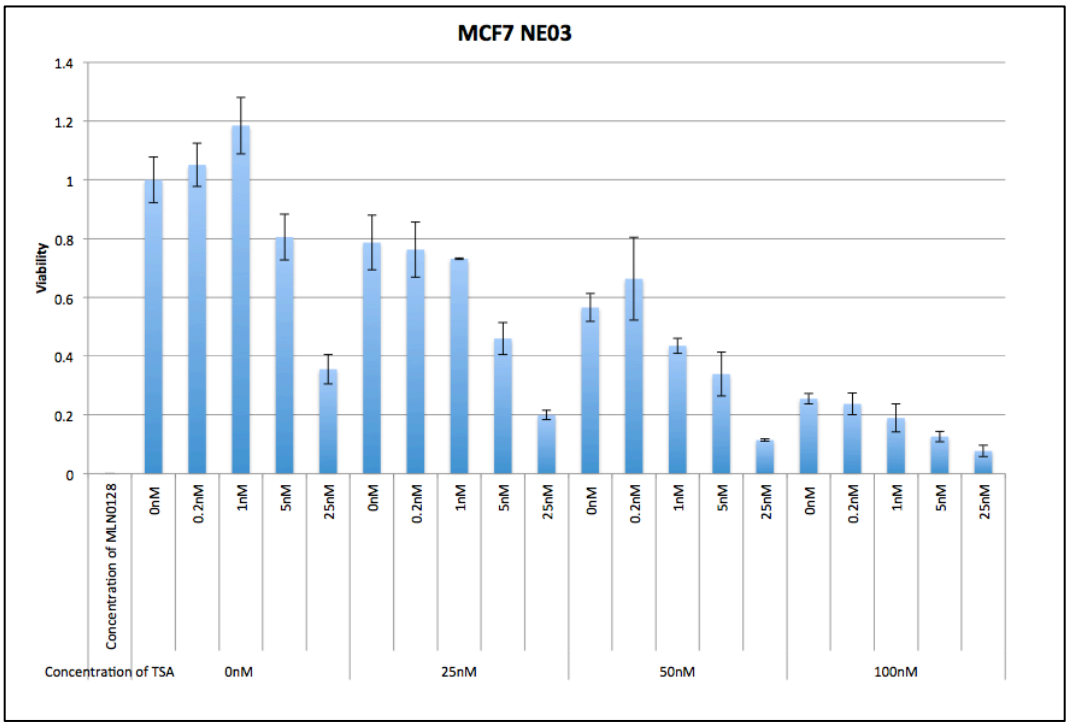
0.2nM
1nM
5nM
25nM
Concentration of TSA
0nM
25nM
50nM
100nM

B


MCF7 NE03
Viability
Concentration of MLN0128
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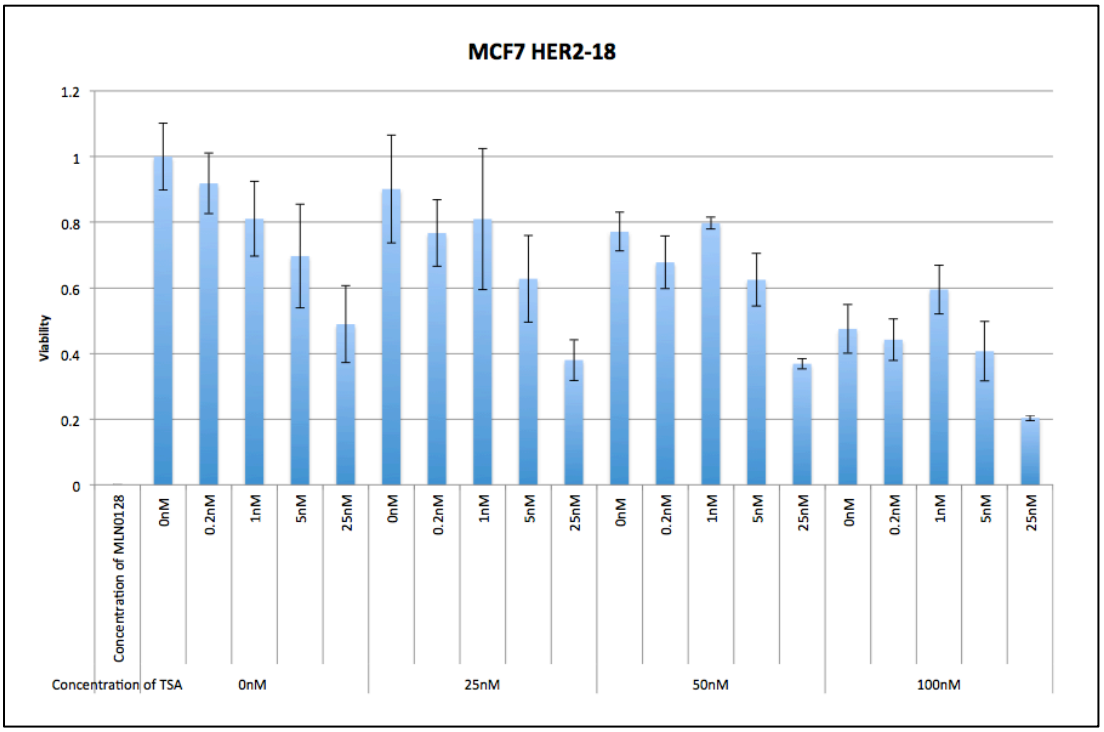
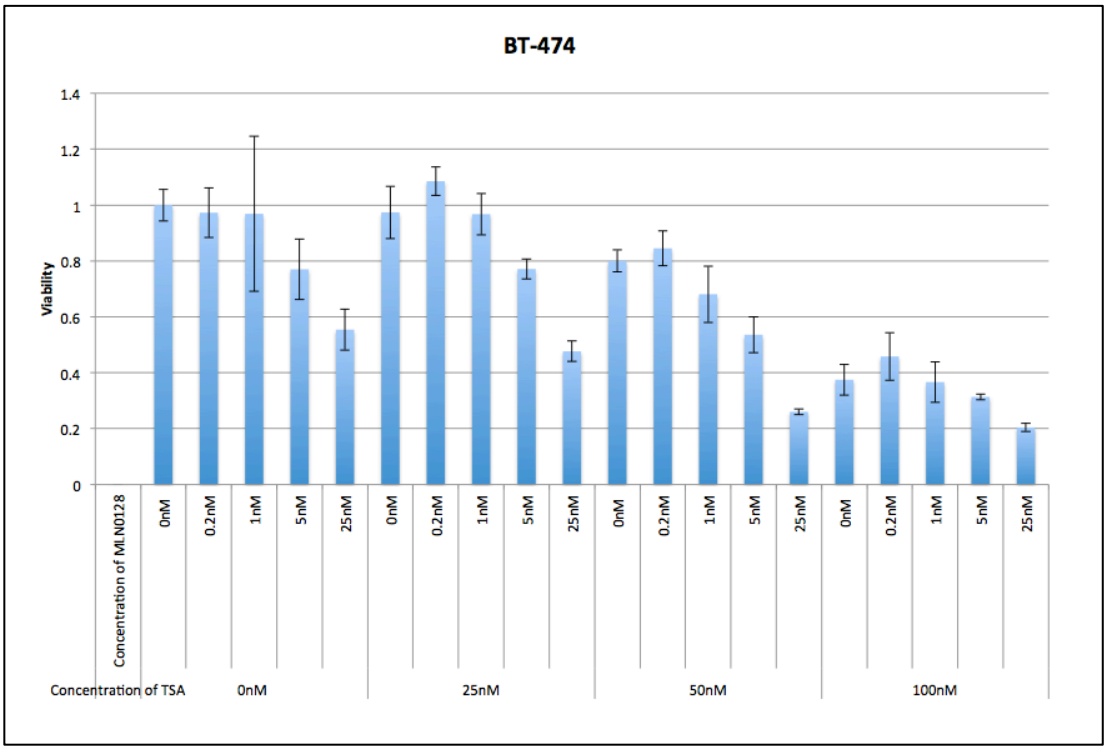
0.2nM
1nM
5nM
25nM
Concentration of TSA
0nM
25nM
50nM
100nM

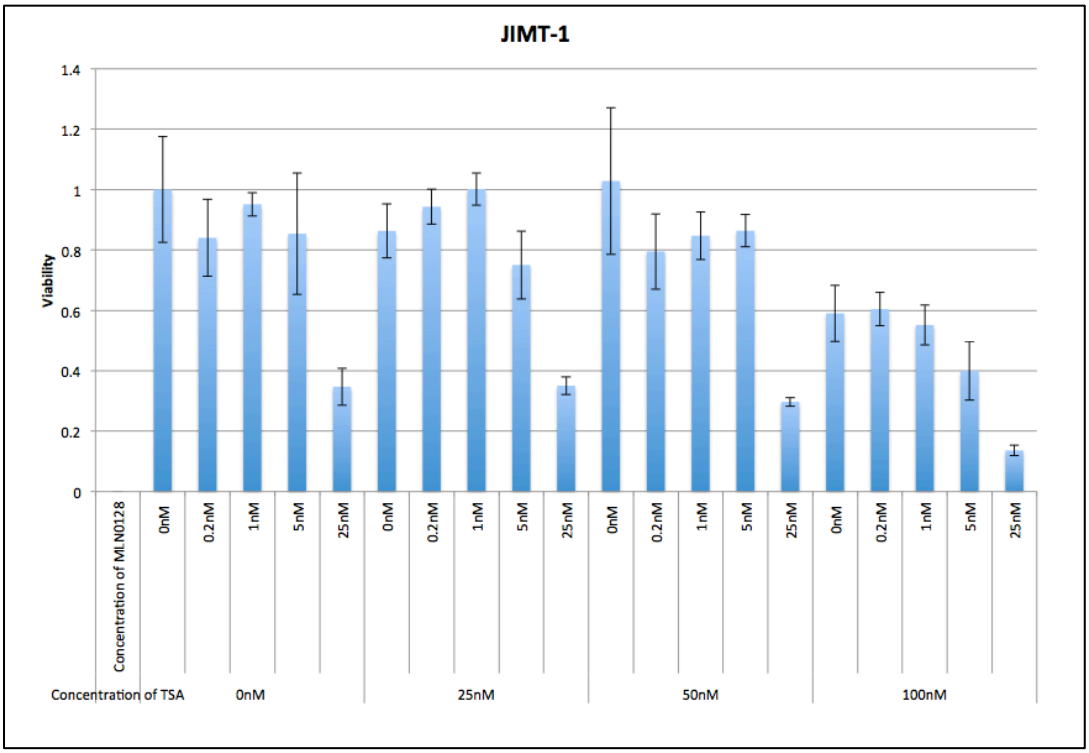
C


MCF7 HER2-18
Viability
1.2
1
0.8
0.6
0.4
0.2
0
Concentration of MLN0128
0nM
0.2nM
1nM
5nM
25nM
Concentration of TSA
0nM
25nM
50nM
100nM

D


BT-474
Viability
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Concentration of MLN0128
0nM
0.2nM
1nM
5nM
25nM
Concentration of TSA
0nM
25nM
50nM
100nM

E


JIMT-1
Viability
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1.2
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0.8
0.6
0.4
0.2
0
Concentration of MLN0128
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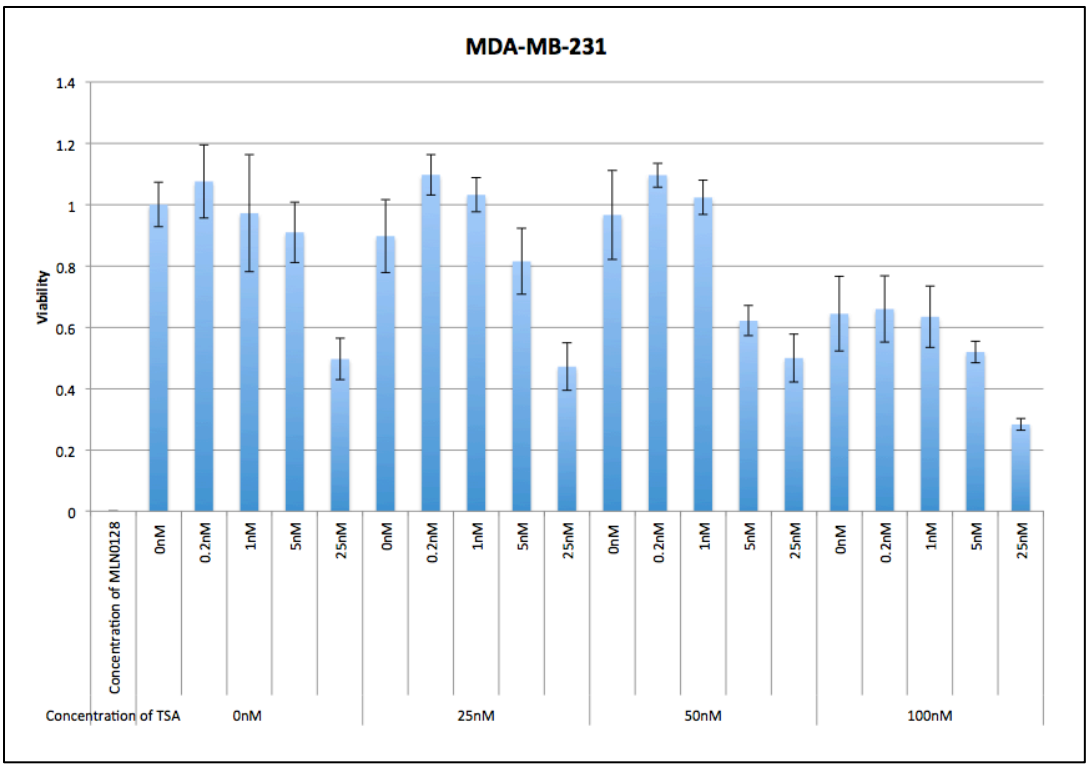
1nM
5nM
25nM
Concentration of TSA
0nM
25nM
50nM
100nM

F


MDA-MB-231
Viability
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Concentration of MLN0128
0nM
0.2nM
1nM
5nM
25nM
Concentration of TSA
0nM
25nM
50nM
100nM

G

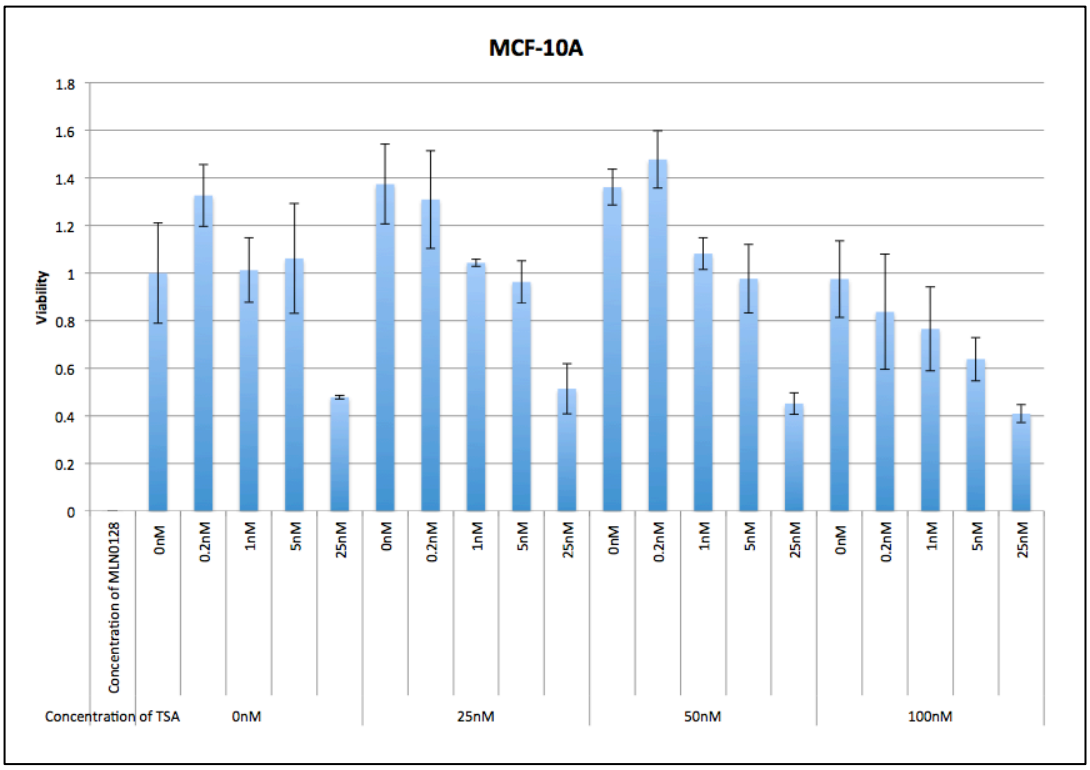


Figure 2. MLN0128 and TSA synergistically inhibit breast cancer cell viability to different degrees in a phenotypically diverse set of breast cancer cell lines. **A.**SKBR3 cells **B.** MCF7 NEO3 **C.** MCF7 HER2-18 **D.** BT-474 **E**. JIMT-1 **F.** MDA-MB-231 **G.** MCF-10A. All cell lines were treated with same increasing concentrations of TSA and MLN0128, in single agent or combination treatments for 3 days. Cell viability assay was performed.

IC_{50} values of MLN0128 and TSA in breast cancer cell lines

Cell Line	ER	PR	HER2	PI3K	PTEN	MLN0-128 IC_{50}	TSA IC_{50}
SKBR3	-	-	+	WT	+	4.64	30.9
MDA-MB-231	-	-	-	WT	+	11.75	89.13
MCF7 NE03	+	+	_	E54K	+	14.1	63.1
MCF7 HER2-18	+	+	+	E54K	+	18.6	>100
JIMT-1	-	-	+	H1047R	-	21.87	>100
BT-474	-	+	+	WT	+	> 25	95.49
MCF-10A	-	-	_	WT	+	25.1	>100

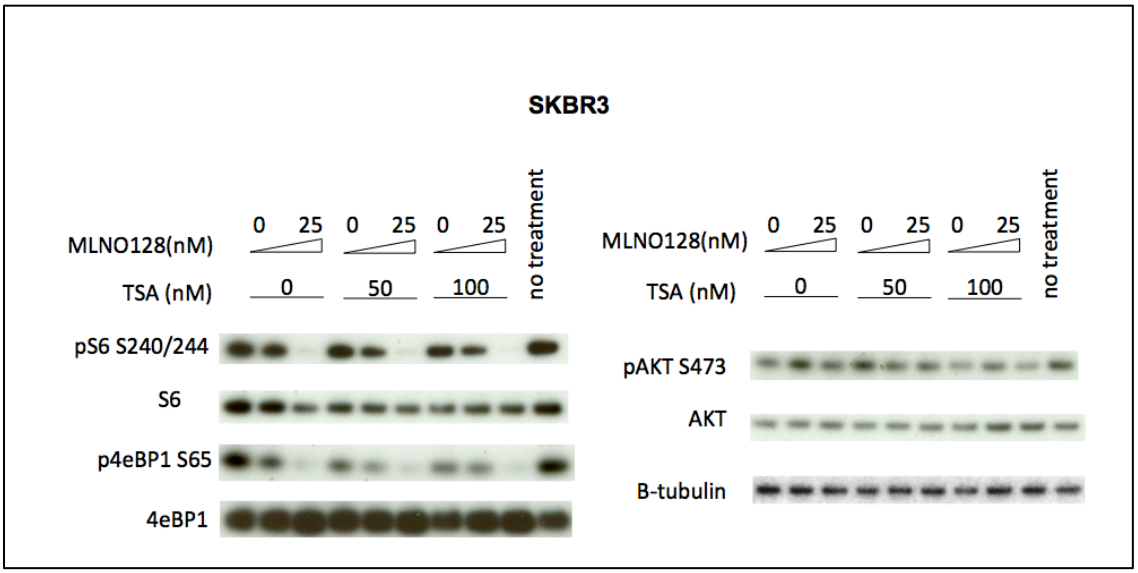
Figure 3. MLN0128 and TSA reduce the viability of a phenotypically diverse panel of breast cancer cell lines with non-transformed MCF10A breast epithelial cells being less affected. IC50 values were calculated for MLN0128 and TSA from each cell line listed following a 3-day cell viability assay.

Synergistic Inhibition of breast cancer cell viability

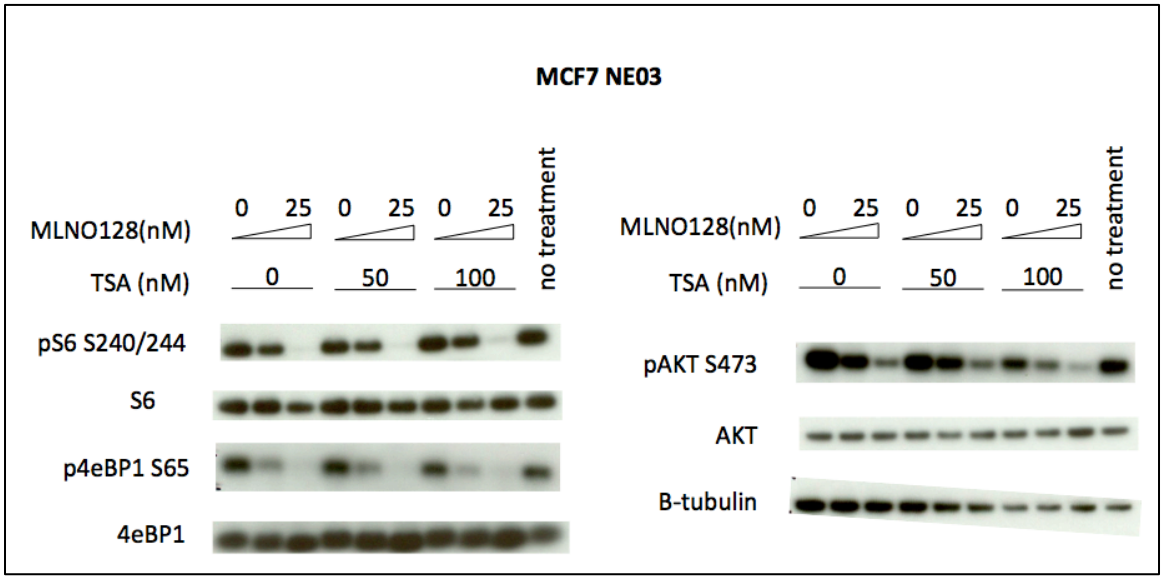
Drug	Drug	Cell line	Cell line	Cell line	Cell line	Cell line
MLN0128 (nM)	TSA (nM)	SKBR3 (CI)	MCF7 NE03 (CI)	MCF7 HER2-18 (CI)	BT-474 (CI)	MDA-MB-231 (CI)
5	50	0.701	0.711	0.742	0.778	4.328
25	50	0.566	0.758	0.903	0.73	0.895
5	100	0.762	0.637	0.873	0.898	0.598
25	100	0.672	0.771	0.811	0.879	0.264

Figure 4. MLN0128 and TSA inhibit breast cancer cell viability in a synergistic manner. Combination index (CI) was calculated in all cell lines where a sufficient effect on viability was observed using the Calcusyn software. Antagonism is indicated by a CI >1.10. Additivity is indicated by a CI ranging from 0.90-1.10, and synergism is indicated by a CI ranging from 0.1-0.90. [43].

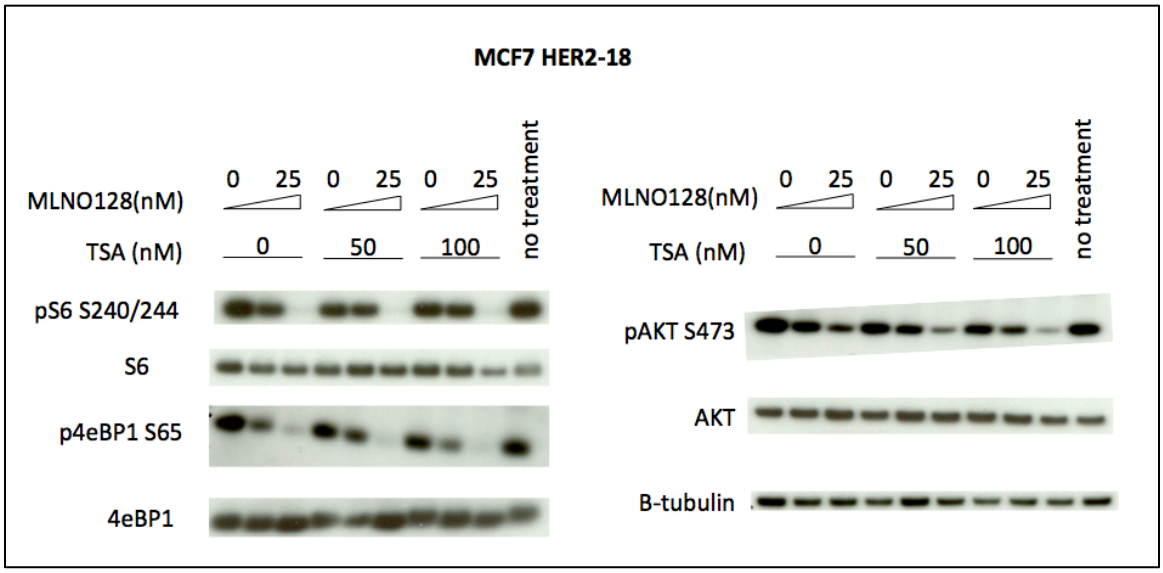
A


SKBR3
MLNO128(nM)
0 25 0 25 0 25
no treatment
TSA (nM)
0 50 100
pS6 S240/244
S6
p4eBP1 S65
4eBP1
MLNO128(nM)
0 25 0 25 0 25
no treatment
TSA (nM)
0 50 100
pAKT S473
AKT
B-tubulin

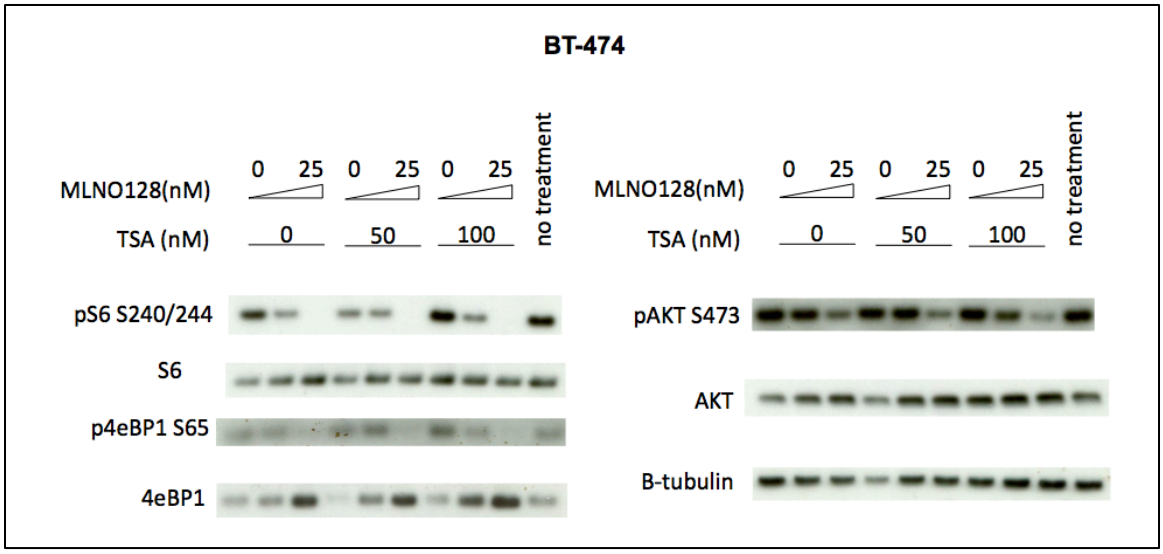
B


MCF7 NE03
MLNO128(nM)
0 25 0 25 0 25
no treatment
TSA (nM)
0 50 100
pS6 S240/244
S6
p4eBP1 S65
4eBP1
MLNO128(nM)
0 25 0 25 0 25
no treatment
TSA (nM)
0 50 100
pAKT S473
AKT
B-tubulin

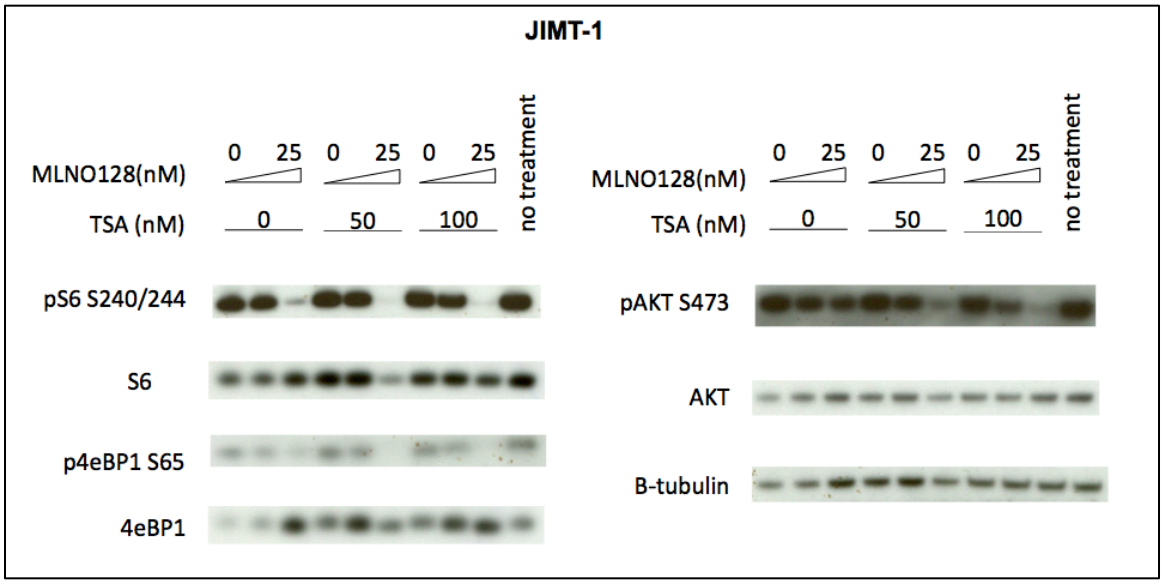
C


MCF7 HER2-18
MLNO128(nM)
0 25 0 25 0 25
no treatment
TSA (nM)
0 50 100
pS6 S240/244
S6
p4eBP1 S65
4eBP1
MLNO128(nM)
0 25 0 25 0 25
no treatment
TSA (nM)
0 50 100
pAKT S473
AKT
B-tubulin

D


BT-474
MLNO128(nM)
0 25 0 25 0 25
no treatment
TSA (nM)
0 50 100
pS6 S240/244
S6
p4eBP1 S65
4eBP1
MLNO128(nM)
0 25 0 25 0 25
no treatment
TSA (nM)
0 50 100
pAKT S473
AKT
B-tubulin

E



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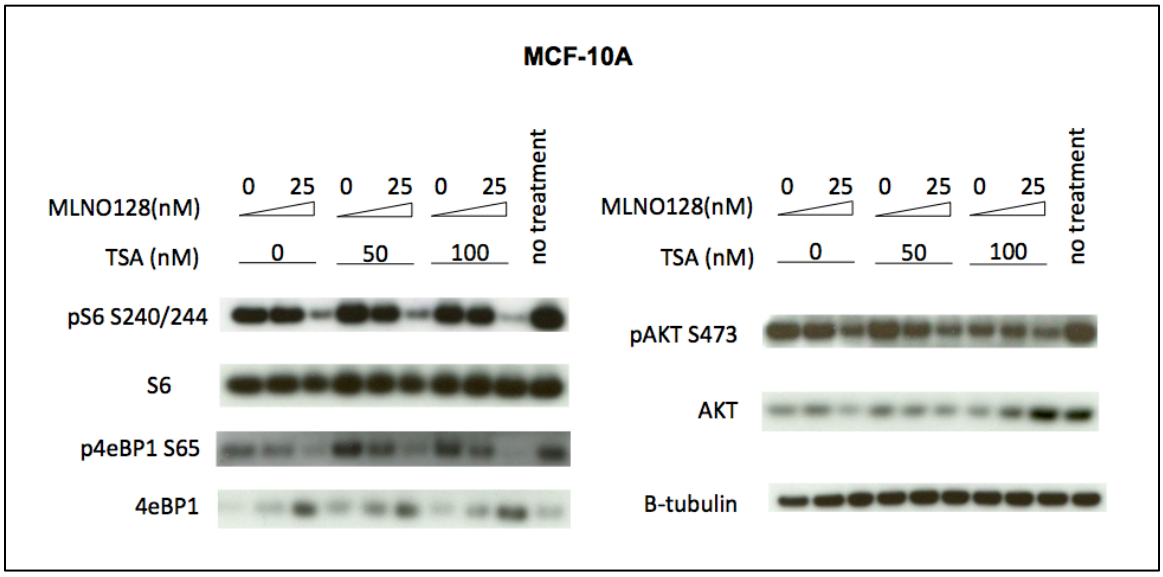

Figure 5. MLN0128 and TSA decrease phosphorylation of downstream effector molecules of mTOR. A. SKBR3 **B.** MCF7 NE03 **C.** MCF7 HER2-18 **D.** BT-474 **E.** JIMT-1 **F.** MCF-10A. Cells were treated with MLN0128 and TSA for 8 hours. Whole cell lysates were collected and western blots were performed using protein specific antibodies.

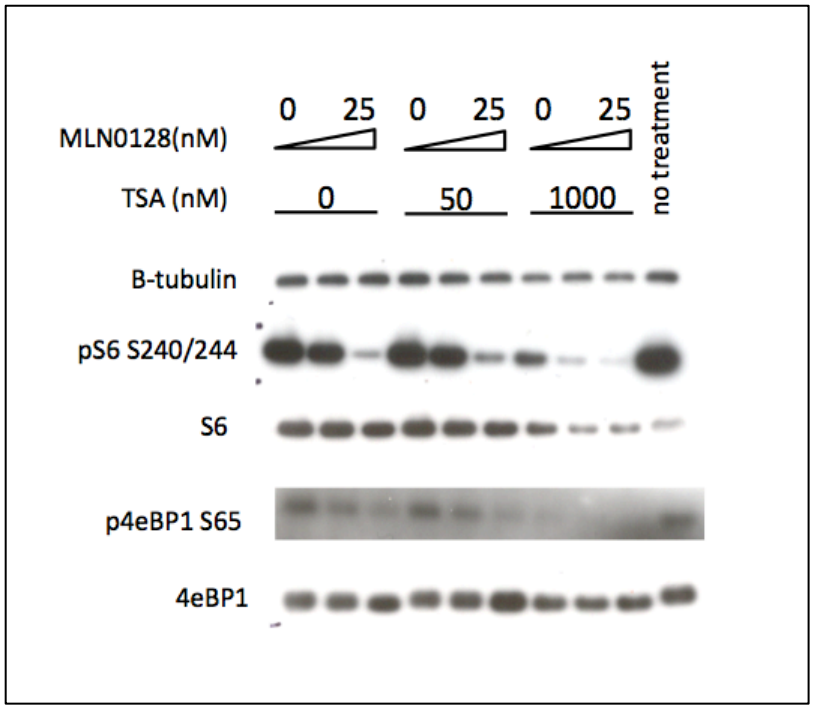

Figure 6. MLN0128 and high doses of TSA reduces pS6 and p4eBP1 levels after 24 hours of treatment. SKBR3 cells were treated with MLN0128 and TSA with increasing concentrations of both drugs as single agents or in combination treatment.

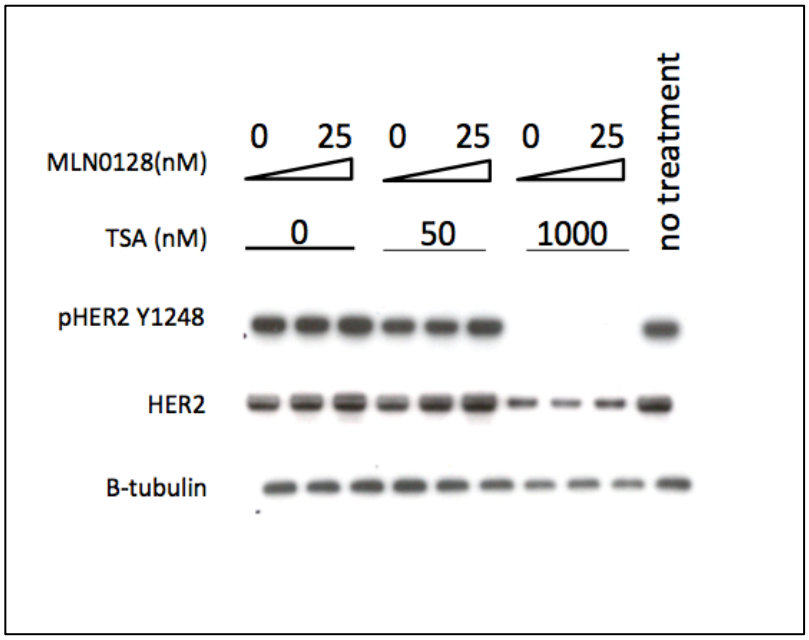

Figure 7. TSA reduces pHER2 and total HER2 protein levels. SKBR3 cells were treated and analyzed as in Figure 6. Beta tubulin was used as a loading control.

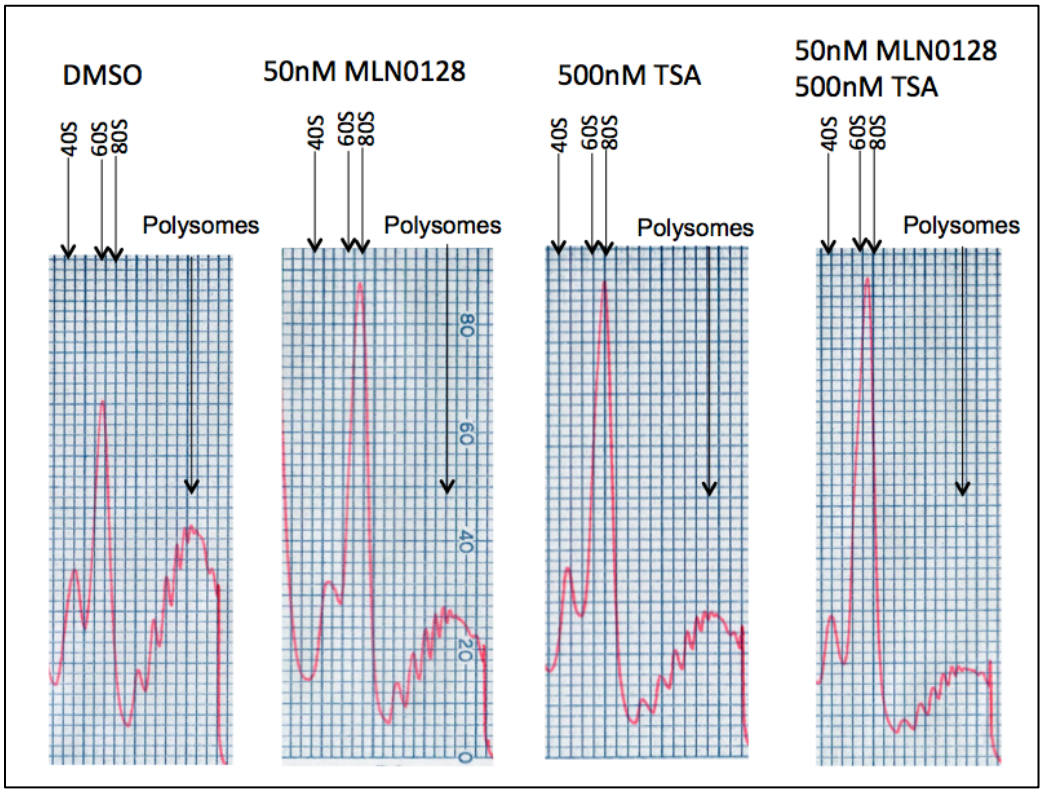

Figure 8. Combination of MLN0128 and TSA decreases polysome formation. SKBR3 cells were treated with a 50nM concentration of MLN0128 and a 500nM concentration of TSA for 24 hours.

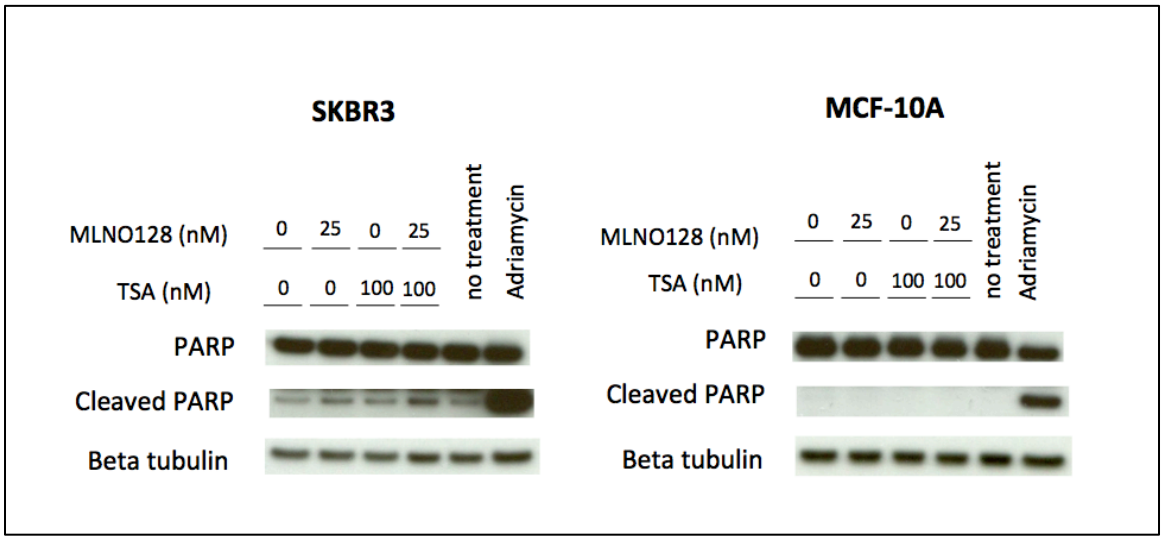

Figure 9. Dual treatment with MLN0128 and TSA induces apoptosis in SKBR3 cells. SKBR3 and MCF-10A cells were treated with MLN0128 and TSA for 48 hours with adriamycin treated cells as a positive control. Whole cell lysates were collected for western blot analysis to probe for cleaved PARP as a marker for apoptosis.

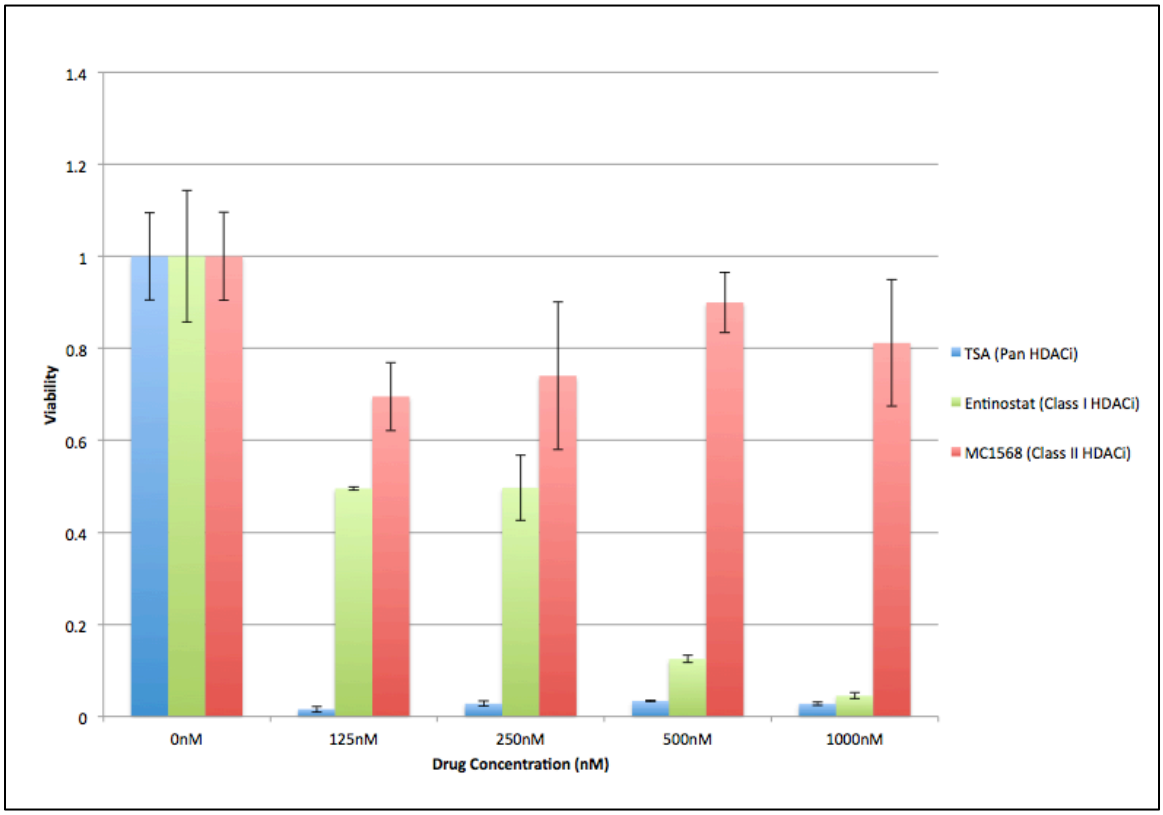

Figure 10. Class I HDAC inhibitor showed greater reduction in breast cancer cell viability compared to class II HDAC inhibitor. SKBR3 cells were treated with TSA, Entinostat, or MC1568 for 3 days. Cell viability assay was performed.

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