Pharmacological inhibition of NOS activates ASK1/JNK pathway augmenting docetaxel-mediated apoptosis in triple negative breast cancer

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Inducible nitric oxide synthase (iNOS) upregulation is associated with chemotherapy resistance in TNBC patients. In the present study, we describe how the addition of a pan-NOS inhibitor, NG-Monomethyl-L-arginine (L-NMMA), can improve docetaxel response by redirecting cell fate from a pro-survival state, driven by endoplasmic reticulum (EnR) stress response, to an apoptotic state via activation of ASK1/JNK pathway. Coupling chemotherapy with NOS inhibition therapy may represent an effective therapeutic alternative for patient with TNBC who have failed conventional therapy.

Abstract

Purpose: Chemoresistance in triple negative breast cancer (TNBC) is associated with the activation of a survival mechanism orchestrated by the endoplasmic reticulum (EnR) stress response and by inducible nitric oxide synthase (iNOS). Our aim was to determine the effects of pharmacological NOS-inhibition on TNBC.

Experimental Design: TNBC cell lines, SUM-159PT, MDA-MB-436, and MDA-MB-468, were treated with docetaxel and NOS-inibitor (L-NMMA) for 24, 48 and 72 hours. Apoptosis was assessed by flow cytometry using Annexin-V and propidium iodide. Western Blot was used to assess ER-stress and apoptosis; rtPCR, to evaluate s-XBP1. TNBC patient derived xenografts (PDXs) were treated either with vehicle, docetaxel, or combination therapy (NOS-inhibition + docetaxel). Mouse weight and tumor volumes were recorded twice weekly. Docetaxel concentration was determined using mass spectrometry. To quantify proliferation and apoptosis PDX tumor samples were stained using Ki67 and TUNEL assay.

Results: *In-vitro*, L-NMMA ameliorated the iNOS upregulation associated with docetaxel. Apoptosis increased when TNBC cells were treated with combination therapy. In TNBC PDXs, combination therapy significantly reduced tumor volume growth and increased survival proportions. In the BCM-5998 PDX model, intratumoral docetaxel concentration was higher in mice receiving combination therapy. Coupling docetaxel with NOS-inhibition increased EnR-stress response via co-activation of ATF4 and CHOP, which triggered pASK1/JNK proapoptotic pathway, promoting cleavage of caspases 3 and 9.

Conclusion*:* iNOS is a critical target for docetaxel resistance in TNBC. Pharmacological inhibition of NOS enhanced chemotherapy response in TNBC PDX models. Combination therapy may improve prognosis and prevent relapse in TNBC patients who have failed conventional chemotherapy.

Introduction

Approximately 40,000 women with metastatic breast cancer die in United States every year, due to treatment failure or treatment resistance (1). Triple negative breast cancer (TNBC) comprises 15% of all breast cancers and patients have higher recurrence, more distant metastases, and worse mortality rates than other breast cancer types (2). TNBC is characterized by the lack of estrogen, progesterone, and HER2 receptor expression. TNBC is a heterogeneous disease without an FDA-approved targeted therapy. Therefore, it is important to differentiate TNBC subtypes and to identify therapeutic targets when treating specific patient subpopulations (3,4). Most TNBC are sensitive to systemic chemotherapies such as taxanes, anthracyclines, and platinum derivatives, yet local and systemic relapses rates are high (2,5).

Resistance to conventional chemotherapies has been correlated with the presence of subpopulations of breast cancer cells with stem-like properties (6,7). Our group described a treatment-resistant signature of 477 genes derived out of TNBC biopsies from chemotherapy treated patients (7). The top genes were analyzed and knockdowns of Ribosomal protein L39 (RPL39) and Myeloid Leukemia Factor 2 (MLF2) were associated with a decrease in nitric oxide (NO) signaling (8), in particular inducible nitric oxide synthase (iNOS, NOS2). RPL39 is a structural protein of the ribosome at its polypeptide exit tunnel, a protein-sensitive channel that regulates translation through recognition of specific sequences (9,10). MLF2 is involved in chromosomal arm 12p aberrations associated with acute leukemias of lymphoid and myeloid lineage (11). NO and reactive NO-derived species have been implicated in the modulation of carcinogenesis and as a critical determinant of oxidative stress in cells (12,13). In TNBC, increased iNOS expression is related to tumor grade, aggressiveness, and poor prognosis (14-16). *In vitro*, iNOS inhibition diminished cell proliferation, cancer stem cell self-renewal, and cell migration in TNBC cell lines. These effects have been replicated in corresponding cell lines in *in vivo* xenografts (16). Additionally, mutations in RPL39 (A14V) and MLF2 (R158W) have been shown to enhance migration in *in vitro* experiments (17). Breast cancer patients harboring RPL39 (A14V) and MLF2 (R158W) mutations demonstrate a shorter median time to relapse with lung metastases, compared to those without these mutations (17). In metaplastic breast cancer, a subtype mutation in RPL39 (A14V) has been correlated with higher levels of iNOS, increased metastatic relapse in the lung, and worse overall survival (18).

NO is a common denominator of the adaptive endoplasmic reticulum (EnR) stress response pathway that results in treatment resistance (19). EnR stress response activates different pathways that promote cell survival under stressful conditions. When cells are unable to overcome these conditions, an apoptotic response is then initiated (19,20). Apoptosis signal-regulating kinase 1 (ASK1) is a member of the mitogen-activated protein kinase kinase kinase (MAP3K) family. ASK1 activates c-Jun N-terminal kinase (JNK) in response to a variety of stress stimuli (21). ASK1 serves as a central signaling hub that mediates EnR stress response and apoptosis (22). Therefore, ASK1's activity is tightly regulated via phosphorylation sites (22). For instance, phosphorylation of Thr845 ASK1 is essential for ASK1 activation, which promotes apoptotic cell death (23). Ser83 remains phosphorylated under low-stress conditions, keeping ASK1 inactive (24,25). Ser967 serves as a sensor that mediates the physical interaction of 14-3-3 with ASK1, which suppresses ASK1 mediated apoptosis (22,26). Because of this, ASK1 has been described as a mediator of the apoptotic cell death resulting from chemotherapy (27).

The aim of this work was to examine whether pharmacological inhibition of NOS signaling could help overcome treatment resistance in TNBC. First, we detected an increase in antitumor activity when docetaxel was combined with a pharmacologic NOS inhibitor, L-NMMA in three TNBC cell lines and five different TNBC patient derived xerographs (PDXs). Then, we examined the cross-talk between NO and EnR stress pathways; NOS inhibition affected the expression of EnR stress-related markers IRE1α, CHOP, and ATF4, causing an increase in apoptosis, identified by activation of caspases 3 and 9, and the ASK1/JNK pathway. Combining chemotherapy with NOS inhibition represents a promising therapeutic opportunity for patients with TNBC, especially for patients with high levels of intratumoral iNOS expression due alterations on MLF2 and RPL39.

Materials and Methods

Reagents

For *in vitro* and *in vivo* experiments, Tilarginine Acetate (L-N-monomethyl arginine) (L-NMMA) pan-NOS inhibitor was purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and diluted in DPBS. For *in vitro* experiments, docetaxel was obtained from Sigma Aldrich and diluted in dimethyl sulfoxide. For *in vivo* studies, docetaxel and amlodipine were purchased

through Houston Methodist Hospital pharmacy and dissolved on DPBS. iNOS (N-20), CREB-2/ATF4 (C-20), CHOP/GADD 153 (B-3), and pASK1 (Thr 845) antibodies were obtained from Santa Cruz Biotechnology (Dallas, Tx). Antibodies IRE1α (14C10), cleaved-caspase 3 (D315), cleaved-caspase 9 (D175), Phospho-SAPK/JNK (Thr183/Tyr185) (81E11), SAPK/JNK, ASK1, Phospho-ASK1 (Ser83), Phospho-ASK1 (Ser967), β-Actin (13E5), anti-rabbit, and anti-mouse IgG were purchased from Cell Signaling Technology (Danvers, MA, USA). IRE1α (phospho S724) and Ki67 were bought from Abcam (Cambridge, MA).

In vitro experiments

Triple negative breast cancer cell lines MDA-MB-468 and MDA-MB-436 were purchased from American Type Culture Collection (Manassas, VA, USA), while SUM-159PT was obtained from Asterand Bioscience (Detroit, MI, USA); no authentication of the cell lines was performed by the authors. All cells were maintained in DMEM (Invitrogen, Grand Island, NY) supplemented with 10% FBS (Thermos Scientific Hyclone, Rockford, IL) and 1% antibiotic-antimycotic in a humidified incubator at 37°C with 5% CO_2. Unless otherwise specified, cells were treated with docetaxel 5nM on day 1, and daily with L-NMMA 4mM.

Western blot analysis

Whole cell lysates were prepared in 1X lysis buffer (Cell Signaling Technology) with 1X protease inhibitor (GenDepot) and 1X phosphatase inhibitor (GenDepot). Samples (30μg protein) were boiled in LDS sample buffer (Thermo Fisher Scientific) containing 1x Sample Reducing Agent (Thermo Fisher Scientific) and subjected to SDS-PAGE electrophoresis in 4% to 12% gradient polyacrylamide gels (Thermo Fisher Scientific). Proteins were transferred onto nitrocellulose membranes (Bio-Rad). Membranes were incubated overnight at 4°C with primary antibodies (1:1,000) followed by incubation with appropriate secondary antibodies for 1 hour (1:2,000). Protein bands were developed in autoradiography films (Denville Scientific Inc., South Plainfield, NJ, USA).

Flow cytometry analysis

Triple negative breast cancer cell lines MDA-MB-436, SUM-159PT and MDA-MB-436 were treated with docetaxel 5nM on day 1, and daily with L-NMMA 4mM for 48 and 72 hours.

Cells were washed, detached, and stained with Annexin V Apoptosis Detection Kit FITC (Ebioscience) according to manufacturer instructions. Flow analysis was performed at the Houston Methodist Research Institute Flow Cytometry Core, using BD FACS Fortessa for acquisition of data and FACS Diva (BD Biosciences) for analysis.

In vivo experiments

All animal procedures have been approved by the Houston Methodist Hospital Research Institute Animal Care and Use Review Office. *In vivo* experiments were conducted in five different human triple negative (estrogen receptor/progesterone receptor/HER2 negative) breast cancer patient-derived xenografts (PDXs) including BCM-2147, BCM-5998, BCM-3107, and BCM-4664. PDXs were derived from primary human breast cancers were transplanted into the cleared mammary fat pad of SCID Beige mice (Envigo, Indianapolis, IN). PDX HM-3818 was derived from an ascites biopsy of a patient with TNBC and expanded by transplantation into the mammary fat pad of SCID Beige mice. When the tumors reached an average tumor volume between 150–250 mm^3, mice were randomized and divided into groups. Mouse weight was recorded and tumor volumes were measured and calculated (0.5 × (long dimension) × (short dimension)^2) twice weekly. Tumor volume fold change was calculated by dividing the average of the last measurement by the initial tumor volume average. Regimen treatment design followed three, two-weeks cycles of docetaxel [20 mg/kg or 33 mg/kg intraperitoneal on day 1] and NOS inhibition therapy from days 2-6 and 9-13 [L-NMMA (400 mg/kg oral gavage on day 2 and 9, 200mg/kg on days3-6 and 10-13) + amlodipine (10 mg/kg intraperitoneal injection on days 2-6 and 9-13]. A Ca+ channel blocker, amlodipine, was administrated to counteract the effects of NOS inhibition on blood pressure as previously described (16).

Mutation analysis

Droplet Digital PCR (ddPCR) was performed using a standard protocol with custom RPL39 (A14V) and MLF2 (R158W) ddPCR probes and primers (Bio-Rad Laboratories, Hercules, CA) as previously described (18).

Docetaxel liquid chromatography-tandem mass spectrometry analysis

Blood and tumor tissue were collected from PDX BCM-5998 after 40 days of treatment. Analysis of docetaxel in plasma and tissues was performed using a chromatography-tandem mass spectrometry method based on a previously established method (28,29).

One step RT-PCR analysis of spliced XBP1

cDNA was synthetized from total RNA and subsequently amplified using MyTaq™ One-Step RT-PCR Kit (Bio-line). The primers were s-XBP1 (5′- CCTGGTTGCTGAAGAGGAGG-3′ and 5′-CCATGGGGAGATGTTCTGGAG3′) and β-Actin (RT² qPCR Primer Assay for Human ACTB: PPH00073G, from QIAGEN). RT-PCR conditions were 1 cycle at 45°C for 30 minutes, 1 cycle of 95°C for 1 minute, and 40 cycles of 10 seconds at 95°C, 10 seconds at 60°C, and 30 seconds at 72°C, followed by 1 cycle at 4°C for 1 hour. cDNA amplicons were resolved in 2% agarose.

Ki67 and apoptotic index

Tumor tissue collected from PDX BCM-5998 after 40 days of treatment was fixed in formaldehyde overnight and then transferred to 70% ethanol. Tumor tissues were processed and embedded in paraffin. After antigen retrieval (Tris-Cl, pH 9.0), paraffin-embedded sections of xenograft tumors were incubated for 1 hour at room temperature with Ki67 (1:100) antibody (Abcam). Paraffin-embedded sections were stained with Click-iT™ Plus TUNEL Assay for *In Situ* Apoptosis Detection, Alexa Fluor™ 647 dye (Thermo Fisher) according to the manufacturer protocol. DAPI (Thermo Fisher) was used as a counter stain. To quantify the apoptotic index, from each sample, 10 different 20x fields were photographed using Nikon Eclipse 90i microscope system, fluorescent intensity was measured with Nikon Elements software, and the apoptotic index was calculated as the average of the sum intensity of each field recorded.

Statistical Analysis

Two-tailed Student's t-test was performed for comparisons between two groups. One-way ANOVA was performed for multiple group comparisons. Two-way ANOVA was used for all animal experiments. To account for multiple comparisons, Tukey's multiple comparison tests for one-way ANOVA and Boneferroni post tests for two-way ANOVA were performed with

Graphpad Prism 5.0 (Graphpad Software Inc., La Jolla, CA, USA). All data were tested for normal distribution, all results represent the mean±SEM of at least five replicate experiments, unless otherwise specified. In all cases, a two-tailed *p* values < 0.05 were considered statistically significant.

Results

Coupled effects of chemotherapy and NOS inhibition

iNOS derived NO has been shown to be related to taxane resistance (30). To further describe this effect, we examined the expression of iNOS and eNOS in the presence of docetaxel over a period of 72 hours in three different TNBC cell lines (SUM-159PT, MDA-MD 436 and MDA-MB 468). Docetaxel induced iNOS in a bell-shaped distribution; the highest expression was noted around 12-24 hours and by 72 hours the levels were similar to basal expression (Supplemental Figure 1). eNOS expression in MDA-MB 436 and MDA-MB 468 remained similar over the course of the treatment; SUM-159PT showed a minimal increase at 48 hours (Supplemental Figure 1). These results suggest that iNOS may be the main NOS upregulated in TNBC, in response to docetaxel. L-NMMA has been previously used in TNBC cell lines to decrease iNOS production of total nitrites (16,31). We evaluated the effect of L-NMMA on docetaxel-induced iNOS. TNBC cell lines were exposed to docetaxel with and without the presence of L-NMMA, then the expression of iNOS was determined by western blot. Similar to previous observations, administration of docetaxel increased the levels of iNOS after 24 and 48 hours in MDA-MB 436 and SUM-159PT (Fig.1A-B), respectively. While L-NMMA alone had minimal effect on iNOS levels, the combination treatment (docetaxel + L-NMMA) blocked docetaxel-induced increases in iNOS expression and contributed to a reduction in iNOS levels in both cell lines (Fig 1A-B). In MDA-MB 468 cells, a decrease in iNOS was seen at 72 hours in lysates from cells exposed to L-NMMA. Notably, the combination treatment resulted in lower levels of iNOS, compared to the docetaxel treated cells. No changes in eNOS expression were detected following any of the treatments (Supplemental Fig. 1B). Drug-induced cell death was evaluated by flow cytometry. Using Annexin V/PI, L-NMMA alone had moderate effect on cell death, notably after 72h of exposure (Fig. 1C-D). Although solo docetaxel promoted cell death,

these effects were significantly enhanced by the co-administration of L-NMMA after 72 hours of treatment (Fig. 1C-D and Supplemental Fig. 2B). Together, these results suggest that docetaxel + L-NMMA can help reduce docetaxel-induced increases in NOS in TNBC cell lines, an effect that may have potential for therapeutic benefit.

Enhanced efficacy of chemotherapy by therapeutic NOS inhibition in TNBC patient-derived xenografts (PDXs).

The effects of pharmacological NOS inhibition in combination with docetaxel were further evaluated using TNBC PDX models. The PDX models were selected based on their different tumor growth rate and response to docetaxel. BCM-5998 has the MLF2 R158W mutation, while the RPL39 A14V mutation is present in BCM-4664. The dose of docetaxel was adjusted (higher dose, 33 mg/kg compared to 20 mg/kg) to treat animals harboring the BCM-4664 xenografts due to its chemoresistance at conventional doses, as previously published (32). Patient's characteristics from the PDX models used have been previously reported (32) and summarized in Table 1. The L-NMMA dose used in these studies is comparable to that previously published and now in clinical trials (clinicatrials.gov NCT02834403), where the hypertensive effect of L-NMMA was reversed with the addition of amlodipine (16,33,34). Administration of L-NMMA, amlodipine, or NOS inhibition therapy (L-NMMA + amlodipine) had no effect on growth in BCM-2147 PDX (Supplemental Fig. 3A). We previously showed decreased on tumor volume growth by inhibition of iNOS with L-NMMA in PDX model BCM-4664(18). We then evaluated the effect of these drugs by the addition of chemotherapy on PDX BCM-2147. Consistent with the results observed in cell lines, tumor growth was significantly decreased by docetaxel and, when compared to the vehicle arm (Fig. 2), anticancer activity was not modified by amlodipine. The combination of docetaxel with L-NMMA or NOS inhibition therapy (L-NMMA + amlodipine) showed a significant enhancement in docetaxel cytotoxic effect, no difference was detected between these two arms (Supplementary Fig. 3B). BCM-3107 and BCM-4664 responded to docetaxel, (Fig. 2B-D), while no effect was observed in BCM-5998 PDX (Fig 2A). Importantly, docetaxel + NOS inhibition therapy significantly reduced tumor volume average volume fold change in all four TNBC PDXs tested: BCM-3107, 1 ± 0.1 *vs* 0.5 ± 0.05; BCM-4664, 1 ± 0.3 *vs* 0.2 ± 0.1; BCM-2147, 2.9 ± 0.2 *vs* 1.6 ± 0.1; BCM-5998, 3.9 ± 0.3 *vs* 1.9 ± 0.4 (average volume fold change ± SEM) (Fig. 2A-D). In agreement with these observations, docetaxel + NOS inhibition therapy

dramatically improved the survival rate compared to vehicle and docetaxel alone arms (Fig 2 E-F).

To identify potential mechanisms involved in the interaction of docetaxel and pharmacological NOS inhibition, levels of docetaxel were evaluated in both tumor tissue and blood (plasma) samples from BCM-5998 PDXs collected at the end of the third cycle. We found that the intra-tumoral concentration of docetaxel was 5.3-fold higher in mice receiving docetaxel + NOS inhibition therapy than in those treated with solo chemotherapy (175.9 ± 26.01 ng/ml vs. 26.38 ± 7.285 ng/ml, respectively, n=5, $p< 0.001$), while no detectable plasma docetaxel was found in either group. Importantly, there were no differences in body weights between both groups in any of the treated PDX models (Supplemental Fig 3).

NOS blockade enhances docetaxel-induced apoptosis by augmentation of EnR stress response

Taxane-derived therapies have been linked to activation of the EnR stress response (35,36). To investigate whether interactions between the NOS inhibitor L-NMMA and chemotherapy may have altered this pathway, western blot analysis of SUM-159PT and MDA-MB 436 cell lysates were performed. As shown in Fig. 3, a survival stress response was activated by docetaxel as evidenced by increased expression of pIRE1α at 48 and 72 hours. Chemotherapy coupled with NOS inhibition also elevated CHOP and ATF4 expression, compared to the docetaxel treated cells. Increased levels of CHOP have been correlated with activation of EnR stress response (19,20) while ATF4 is usually related to autophagy survival pathways. However, ATF4-related autophagy is switched to apoptosis by subsequent CHOP upregulation (37). Our data suggests that L-NMMA enhanced the lethality of docetaxel (Fig. 1C-D) as, in the presence of docetaxel + L-NMMA, a marked increase in CHOP was observed.

Furthermore, since ASK1 activation, as a cell death mediator, is a downstream target of pIRE1α (38,39), we evaluated its potential involvement in docetaxel + L-NMMA-induced cell lethality. As shown in Figure 3, docetaxel induced pASK1Ser967 pro-survival upregulation. However, docetaxel + L-NMMA increased phosphorylation of pro-apoptotic site Thr845 on ASK1 while decreasing phosphorylation of inhibitory and pro-survival sites Ser967 and Ser83. Pro-apoptotic activation of ASK1 was associated with increased levels of pJNK and cleaved caspases 3 and 9, compared to those in the docetaxel treated cells (Fig 3 and Supplemental Fig 3). s-XBP1,

another target of pIRE1α, has been shown to be upregulated by chemotherapy (40). We were able to detect a docetaxel-dependent increase in s-XBP1, however its levels remained the same when L-NMMA was added (Supplemental Fig. 4).

Similarly, we analyzed tumor lysate from BCM-5998 PDX that had been treated for 40 days. Importantly, the presence of the NOS inhibition therapy, together with docetaxel, reduced the levels of iNOS (Fig. 5A) and resulted in increased CHOP. Docetaxel + NOS inhibition therapy also activated pro-apoptotic JNK (pJNK); some between-replicate variation was observed in-between the groups, probably due to change in hypoxia levels due to different tumor size. A densitometrical analysis was performed to evaluate the changes on phosphorylation of JNK, IRE1α, and ASK1. No differences were observed on pIRE1α. Importantly, pJNK was significantly increased in the combination group when compared to docetaxel, and pASK1 Thr845 were higher in the docetaxel + L-NMMA group, compared to vehicle; both inhibitory sites pASK1 Ser967 and Ser83 were also up-regulated, probably as a negative feedback to control apoptosis (Supplemental Fig. 5). We also evaluated Ki67 and apoptotic index, as shown in Fig. 4 B and Supplemental Fig. 5. Docetaxel increased the levels of Ki67. However docetaxel + L-NMMA display similar levels of Ki67, compared to the vehicle group. However, the apoptotic index was significantly increased by the combination treatment (docetaxel + L-NMMA). In TNBC patients, an increase in Ki67 post-chemotherapy has been correlated with a worse prognosis (41); here we show combination of docetaxel and pharmacological NOS inhibition can increase docetaxel-related apoptotic activity. Based on these findings, NOS inhibition may cause a switch from a EnR stress pro-survival pathway (induced in response to docetaxel) to a pro-apoptotic course (mediated by co-activating CHOP and ATF4), resulting in an increased phosphorylation of pASK1pThr845 by pIRE1α, and in an induction of pJNK-mediated cell death, as shown by increased levels of cleaved (active) caspases 3 and 9 (represented in Fig 3 B).

Therapeutic NOS inhibition response on a PDX with MLF2 and RPL39 Mutation

In previous studies, we identified the novel genes RPL39and MLF2, and demonstrated their association with stem cell self-renewal, treatment resistance, and lung metastasis in TNBC (8). Moreover, we found that both MLF2 and RPL39 increased iNOS-mediated NO production (8). Mechanistically relevant to these effects were the RPL39 A14V and MLF2 R158W mutations (8). Furthermore, in metaplastic breast cancer, a highly chemotherapy-resistant form of breast

cancer, we determined that the RPL39 A14V mutation and iNOS expression are both associated with reduced patient overall survival (18). To test the efficacy of combining docetaxel and L-NMMA, we used the HM-3818 PDX, which was derived from an ascites sample from a patient with metastatic TNBC (Table 1). Mutations in RPL39 (A14V) and MLF2 (R158W) were identified by ddPCR in the patient's plasma and ascites fluid, and also confirmed in the PDX TNBC-3818. HM-3818 displayed a very aggressive, fast growing phenotype so it was treated with a docetaxel dose of 33 mg/kg. While NOS inhibition therapy alone showed a statically significant reduction in tumor volumes by day 8, tumor continued to grow at a rate comparable to the vehicle-treated control tumors (Fig. 5A). Docetaxel, on the other hand, decelerated tumor growth when compared to the vehicle arm, although no significant difference was observed within their survival proportions (Fig. 5B-C). In contrast, the response of HM-3818 PDX model to docetaxel + NOS inhibition therapy showed a significant improvement over docetaxel- and vehicle-treated groups, which resulted in a significantly better survival rate (Fig 5C). These findings suggests that adding NOS inhibition to chemotherapy-based regimens may prove beneficial for patients, especially in tumors harboring enhanced activation of the NOS pathway as a result of alterations on MLF2 and RPL39 function.

Discussion

Resistance to chemotherapy is a major obstacle in patients with TNBC, due to activation of survival mechanisms related to EnR stress (30,40). RPL39 and MLF2 belong to a set of genes that are upregulated in TNBC in response to chemotherapy (7) and both correlate with iNOS regulation (8). iNOS is an inflammatory mediator (42) capable of promoting the survival and proliferation of different cancer types such as melanoma (43-45), liver (46), colon, head and neck (44,47) and glioblastoma (48). In TNBC and metaplastic breast cancer, iNOS expression levels are correlated with aggressiveness, poor survival, and treatment resistance (8,14,16,18,30). In the present study, we demonstrate that the pan-NOS inhibitor L-NMMA interacts with docetaxel-based chemotherapy to overcome treatment resistance through a mechanism that redirects cell fate towards apoptosis (as opposed to survival) through activation of pro-cell death ASK1/JNK pathway.

Our results are similar to other *in vitro* and *in vivo* studies showing the feasibility of pharmacological NOS inhibition combined with cytotoxic chemotherapy as a treatment for cancer (43,49,50). In this study, we describe the crosstalk between treatment resistance and EnR stress, and targeting NOS signaling may overcome this resistance. Cellular regulation of NOS depends on conditions associated to the tumor microenvironment (51,52). In TNBC cell lines, cytokines, hypoxia, nutrient deprivation, and other metabolic factors establish a feed-forward regulatory loop orchestrated by NOS (30,53). EnR stress occurs in response to nutrient deprivation, hypoxia, and alterations in protein glycosylation, resulting in accumulation of unfolded and/or misfolded proteins in the EnR lumen (19,20). The unfolded protein response (UPR) is one of the cellular defense mechanisms triggered in response to chemotherapy (35,36,54). HIF1α hypoxia related response has also been described as a possible mechanism of resistance activated by chemotherapy (40,54). Human melanoma cells under EnR stress acquire resistance to microtubule-targeting drugs through XBP-1-mediated activation of Akt (55). XBP1 is a substrate for IRE1α and maintains a HIF1α/driven hypoxic response (40), IRE1α promotes unconventional splicing of XBP1 mRNA allowing translation of a functional transcription factor, thereby up-regulating ER chaperones(56). Previously, we showed that iNOS inhibition decreased sXBP1 levels (16). In this study, we also identified how docetaxel produced an increase in pIRE1α and s-XBP1. However, coupling docetaxel with NOS inhibition therapy only resulted in further activation of pIRE1 and IRE-1 serves as adaptor protein for TRAF2 and ASK1 promoting ASK1/JNK apoptotic activity (57,58). Importantly, NO produced by NOS has been shown to bind and nitrosylates ASK1 and JNK, inactivating their apoptotic cascade (59,60). Another mechanism of drug resistance that may be involved is PIM1 activation, which has been shown to be activated by docetaxel (61). PIM1 promotes cell survival by phosphorylating the inhibitory site Ser83 on ASK1 (25) and PIM1 has been correlated with tumor aggressiveness and poor survival in TNBC (62). Combining NOS inhibition therapy with docetaxel may prevent activation of PIM1, resulting in a decrease of pASK1 Ser83 levels, and an increase in pASK1 Thr845 levels, which results in activation of effector proteins JNK and cleaved caspases 3 and 9 (Fig 4 B).

L-NMMA is a pan-NOS inhibitor, affecting NO production from all 3 NOS isoforms (63). The rationale of this study was to block NO production thus attenuating the effect of docetaxel-related up-regulation of iNOS. However, L-NMMA may also inhibit eNOS and nNOS activity. eNOS gene polymorphisms are related with the development of breast cancer (64) and nNOS is related

to the increase of cancer-associated fibroblasts in breast cancer (65). Using a pan-NOS inhibitor may help to overcome NO related carcinogenic effects. iNOS inhibitors, namely ASP9853, have been evaluated in clinical trials (66). However ASP9853 was discontinued due to neutropenia. L-NMMA was proven safety in different clinical trials as a hemodynamic modulator (63), and its currently in trial in combination with docetaxel in metastatic triple negative breast cancer (67). Another limitation of our findings is the use of amlodipine, a Ca^{2+} channel inhibitor, to control high blood pressure related to NOS inhibition. Intracellular Ca^{2+} homeostasis is associated with tumor progression (68). Importantly, the expression of high voltage-activated Ca2+ channels in non-excitable cells is limited, and even if these channels are expressed, they depend depolarization to be activated (69). Amlodipine was shown to be effective on a HT-39 breast cancer line xenograft (70), and to induce apoptosis *in-vitro* in TNBC cell line MDA-231 (71). Its interaction may be related to the activity in other channels, rather than voltage-gated Ca^{2+} (72). In the TNBC PDXs treated for this study, no amlodipine-mediated effects on tumor growth inhibition were observed. That said, a thorough appraisal of the effects and interaction of amlodipine in this setting are out of scope of this study.

Preliminary data in chemotherapy-resistant PDX models support our working hypothesis that inhibition of NO signaling with the pan-NOS inhibitor L-NMMA may effectively reverse the malignant course of therapy-resistant cells, significantly improving treatment outcomes in patients with TNBC. The identification of gain-of-function mutations RPL39 (A14V) and MLF2 (R158W) may serve as screening tools and/or biomarkers that inform researcher and clinicians as to whether they should treat patients who have TNBC with a cytotoxic agent and NOS inhibition therapy. However additional studies are required to validate this proposal. While the role of NOS role in other cancer types has been well characterized, knowledge is very limited regarding the role MLF2 and RPL39 mutations or overexpression. Analyzing their repercussions on other types of cancer that are resistant to chemotherapy may open the possibility for basket clinical trials. In conclusion, coupling chemotherapy with NOS inhibition therapy may represent an effective therapeutic alternative for patient with TNBC who have failed conventional therapy.

Disclosure of Potential Conflicts of Interest

412 The authors declare no competing interests.

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628 **Table 1. PDX's patient characteristics**

Xenograft	Age	Patient Ethnicity	Tumor Source	ER	PR	HER2	K19	p53	CK5/6	EGFR	Patient Clinical Treatment(s)	Patient Clinical Response	Patient Tumor Type	Pam50 Intrinsic Subtype	Pietenpol TNBC Subtype[1]	RPL39 mutation [2]	MLF2 mutation[2]
BCM-2147	33	African American	Pre P.Br	-	-	-	+	-	+	-	AC	Res	IDC	Basal	BL1	-	-
BCM-3107	58	Caucasian	Post P.Br	-	-	-	+	+	+	+	Doc	Sen	IDC	Basal	M	-	-
BCM-4664	nr	African American	Pre P.Br	-	-	-	+	-	-	+	Das + Doc	Res	IDC	Basal	IM	+	-
BCM-5998	nr	Caucasian	Pre CWR	-	-	-	+	-	+	-	AC	Res	IDC	Basal	nd	-	+
HM-3818	58	Hispanic	Met Asc	-	-	-	+	-	+	-	AC/Doc/ Pac	Res	IDC	nd	nd	+	+

629 Abbreviations: Pre, pre-treatment; Post, post-treatment; P.Br, primary breast; CWR, chest wall
630 recurrence; IDC, invasive ductal carcinoma; Asc, Ascities; Met, metastatic disease; AC,
631 doxorubicin (Adriamycin) and cyclophosphamide (Cytoxan) ; Doc, Docetaxel; Pac, Paclitaxel ;
632 Sen, ≥ 30% response; Res, < 30% response; nd, not determined; nr, not reported

633 [1] Pietenpol Classification: BL1, basal-like 1; M, Mesenchymal-like; IM, immunomodulatory; nd,
634 not determined;

635 [2] RPL39 (A14V) and MLF2 (R158W) mutation were evaluated by ddPCR

LEGENDS TO FIGURES

Figure 1. L-NMMA prevents iNOS up-regulation by docetaxel. (**A-B**) MDA-MB 436 and SUM-159PT were treated with L-NMMA (4 mM daily) and/or docetaxel (5 nM on day 1) for 24, 48 and 72 hours, iNOS expression was assessed by western blot. (**C-D**) MDA-MB 436 and SUM-159PT were treated with L-NMMA (4 mM daily) and/or docetaxel (5 nM on day 1) for 48 and 72 hours, cells death was evaluated by flow cytometry using Annexin V and PI staining. (*$p < 0.05$, **$p<0.01$, ***$p<0.001$).

Figure 2. Combination therapy improved docetaxel antitumor effect. (**A-D**) Mice growing orthotopic tumors BCM-5998 (n=10 per arm), BCM-3107 (n=5 per arm), BCM-4664 (n=10 per arm) and BCM-2147 (n=7 per arm) were randomized and treated with vehicle, docetaxel (20 mg/kg or 33 mg/kg), or combination therapy (docetaxel + NOS inhibition therapy [L-NMMA+Amlodipine]). Tumor volumes were measured twice weekly. Average tumor volumes ($0.5 \times$ (mm long dimension) $\times$ (mm short dimension)^2). Data are mean tumor volume ± SEM (**E-F**) Kaplan-Meier survival curves of models BCM-4664 and BCM-2147 treated with vehicle, docetaxel, NOS inhibition therapy (L-NMMA + Amlodipine) and Combination therapy (Docetaxel+L-NMMA+Amlodipine). An event was score when a tumor reached 1200 mm^3. Combination therapy increased survival proportions compared to chemotherapy alone. (*$p < 0.05$, **$p<0.01$, ***$p<0.001$)

Figure 3. NOS inhibition prevents pro-survival pathways activated by docetaxel. SUM-159PT and MDA-MB 436 were treated with L-NMMA (4mM) and/or docetaxel (5nM) for 48 and 72 hours, cell were collected and protein extracted, EnR stress and apoptosis markers were

evaluated with western blot. (**B**) Schematic pathway of the EnR stress and apoptotic response enhanced by addition of NOS inhibition.

Figure 4. NOS inhibition therapy enhances apoptotic response. (**A**) Mice growing orthotopic tumors BCM-5998 (n=5) were randomized and treated with vehicle, docetaxel, or combination therapy (Docetaxel + NOS inhibition therapy), after 40 days of treatment tumors were collected and processed, target engagement, EnR stress and apoptosis markers were evaluated by western blot. (**B**). Tumor slides were stained with Ki67antibody, a representative picture was selected to show differences between treatments, Amplification: 20x; counterstain: hematoxylin. (B). TUNEL assay was performed on paraffin embedded tumor slides, 10 different 20x field were capture per sample, the sum of the intensity was measurement, and the average of each treatment was calculated. (*$p < 0.05$)

Figure 5. HM-3818 Response to NOS inhibition and combination therapy. (**A**) Mice growing orthotopic tumors HM-3818 (n=5 per arm) were treated with vehicle or NOS inhibition therapy, tumor volumes were measured twice weekly. Average tumor volumes ($0.5 \times (\text{mm long dimension}) \times (\text{mm short dimension})^2$). Data are mean tumor volume ± SEM. (**B**) Mice growing orthotopic tumors HM-3818 were sorted and treated with Vehicle (n=4), docetaxel (n=6) and combination therapy (docetaxel + NOS inhibition therapy) (n=6 per arm), tumor growth was assessed as previously noted. (**C**) Kaplan-Meier survival curves of model HM-3818. An event was score when a tumor reached 20000 $mm^{3.}$ Combination therapy increased survival proportions compared to chemotherapy alone. (*$p < 0.05$, ***$p<0.001$)

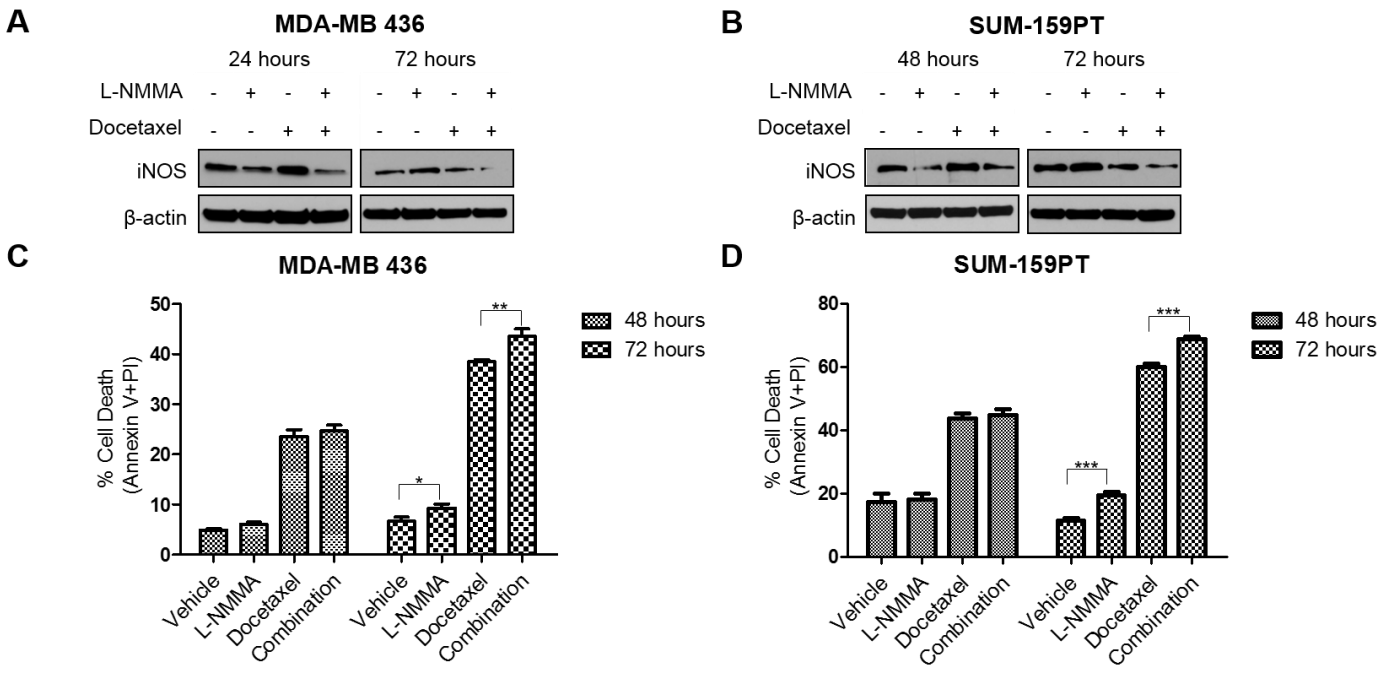


Figure 1

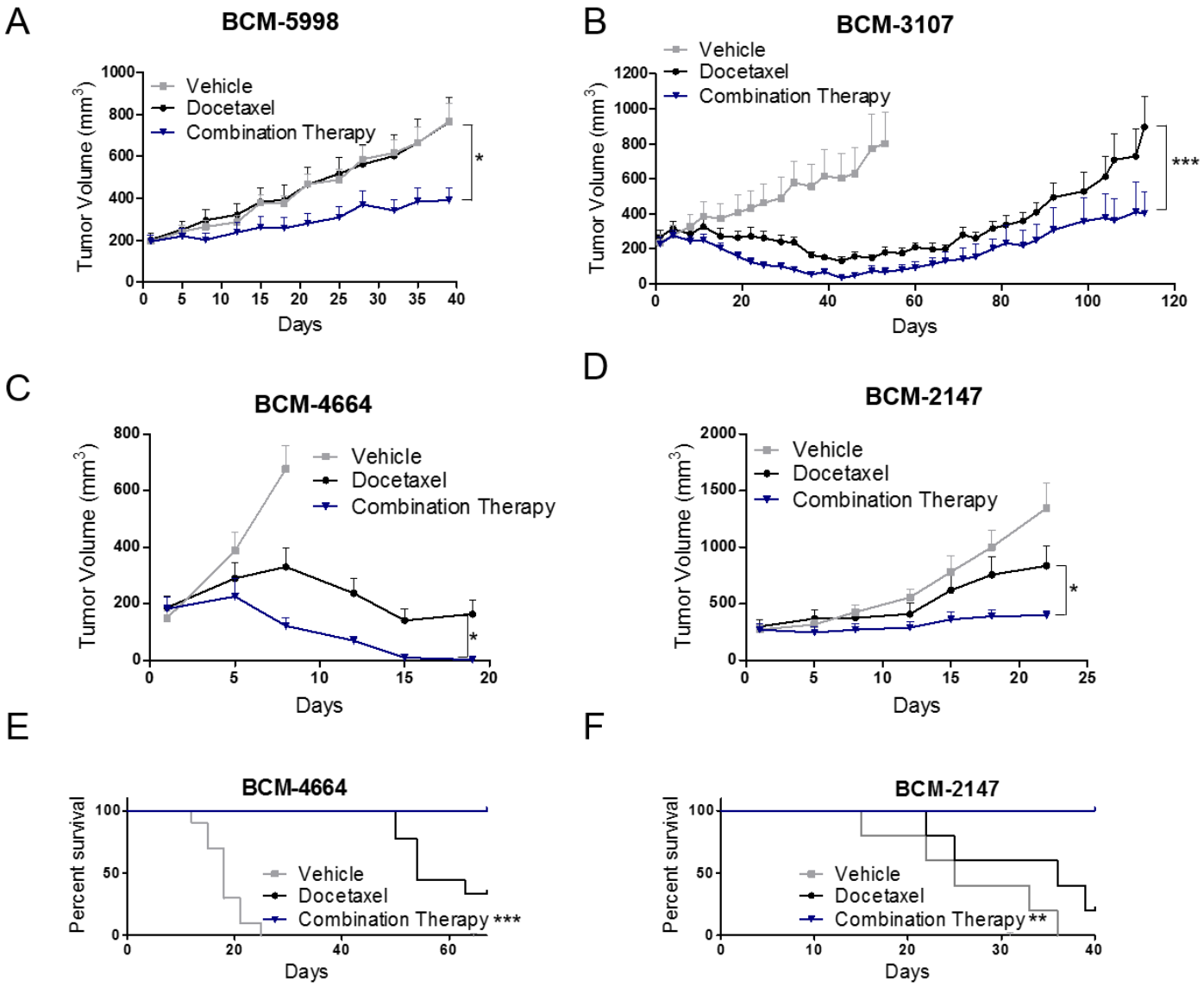
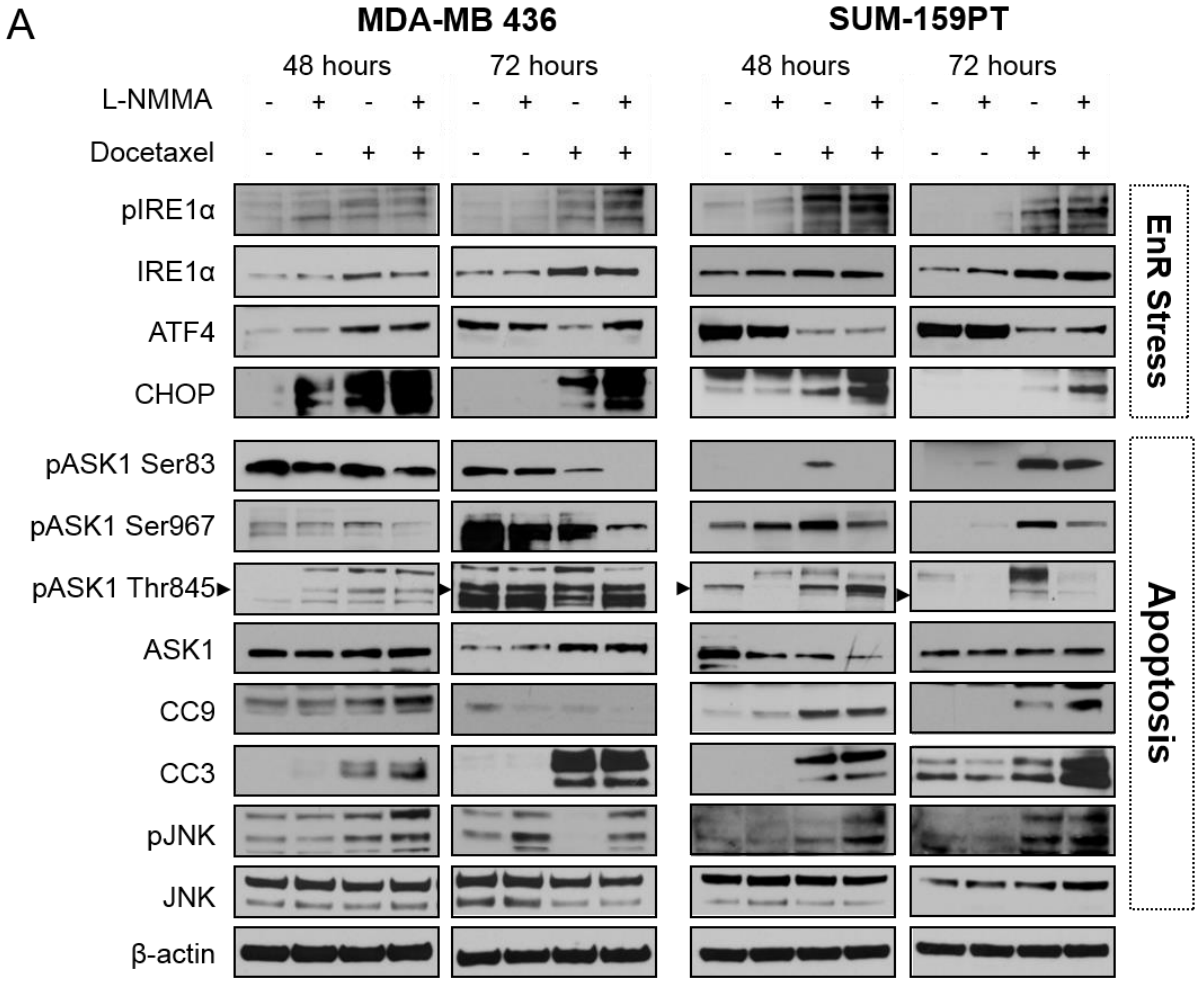


Figure 2



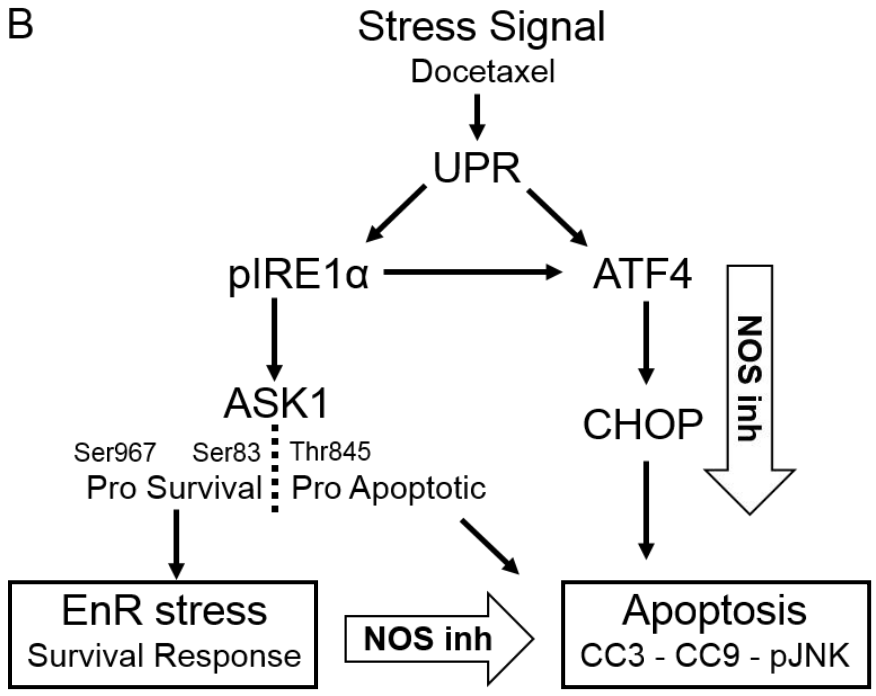


Figure 3

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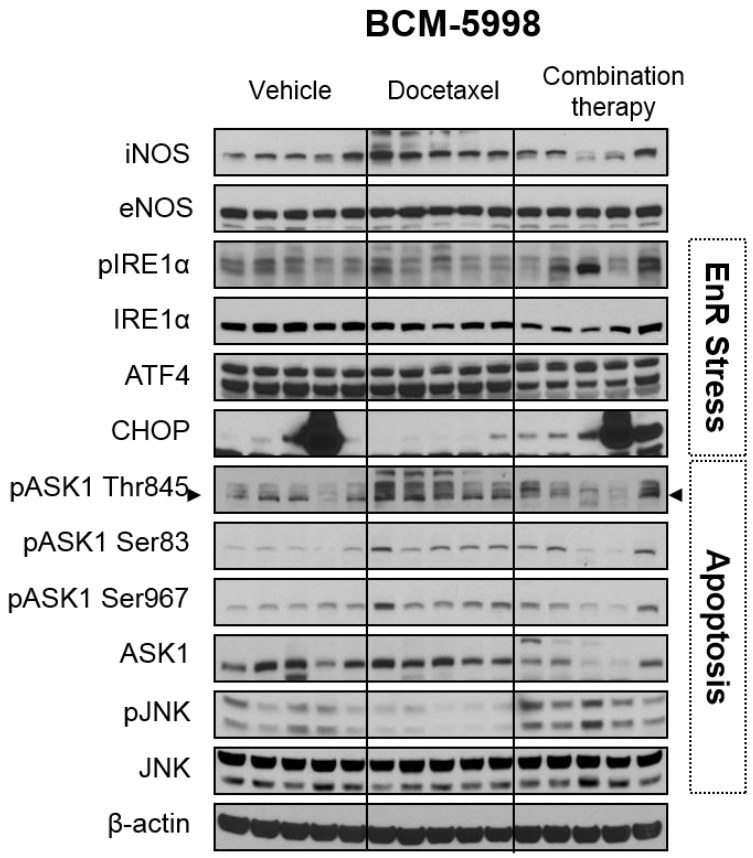

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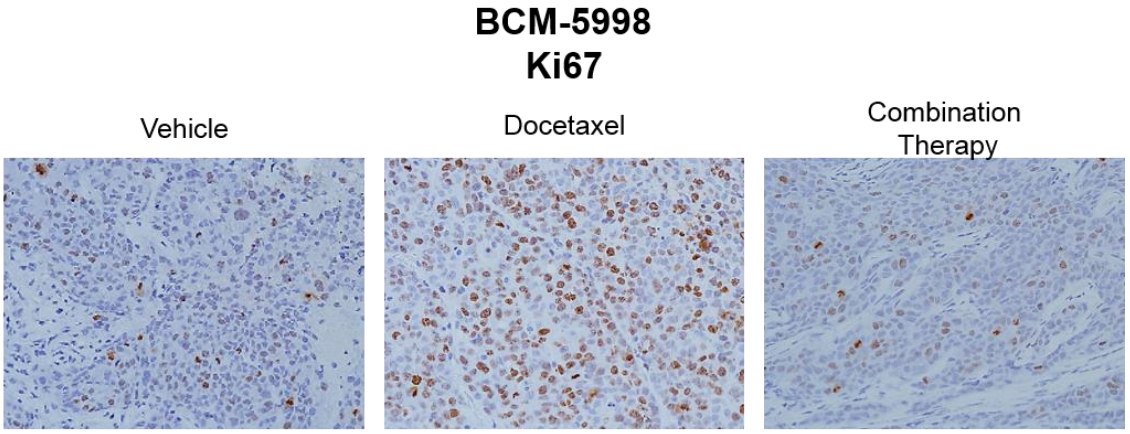

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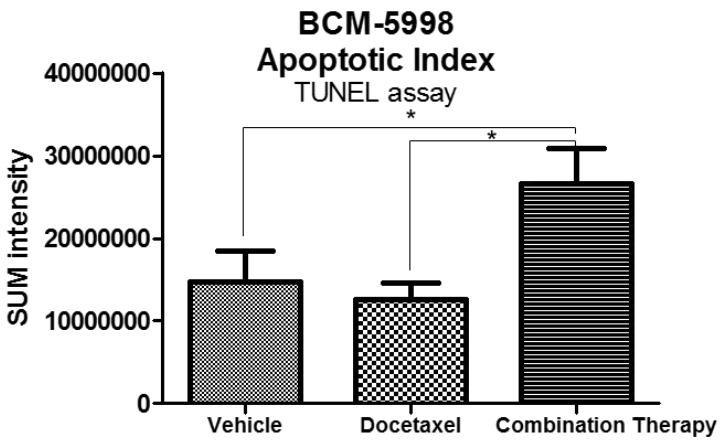
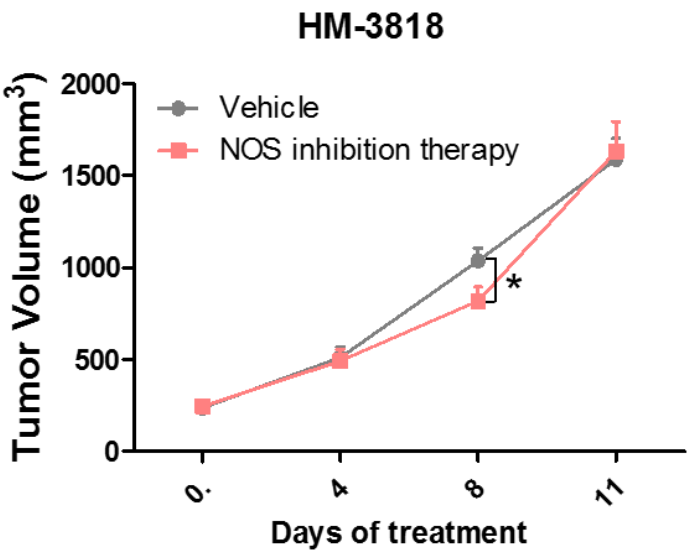


Figure 4

A



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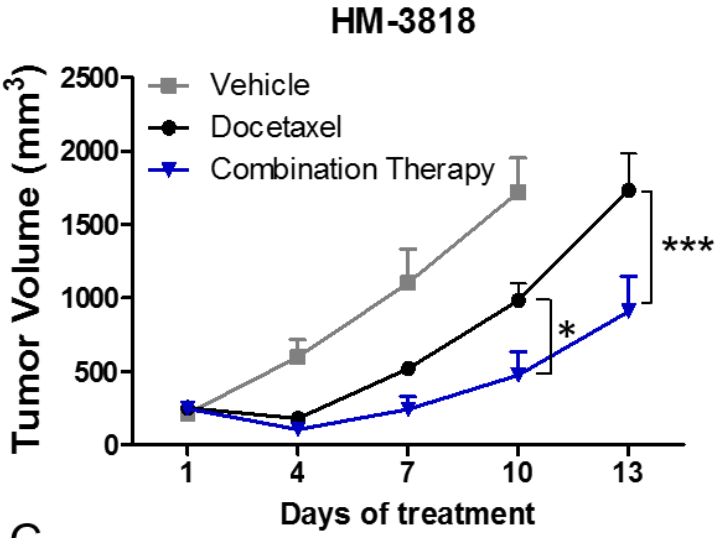

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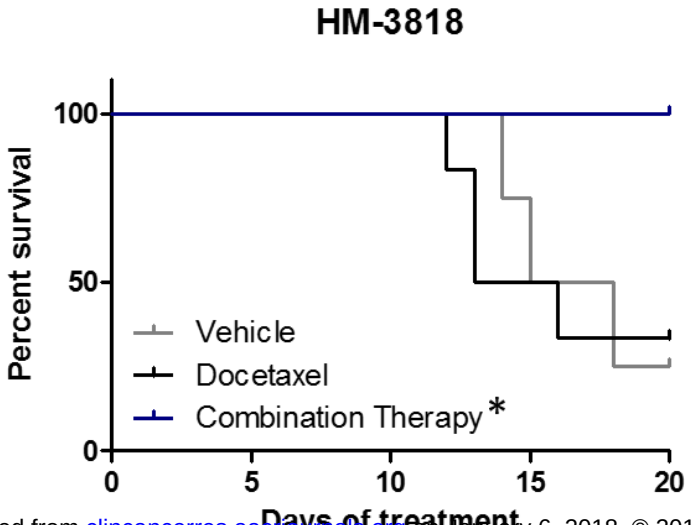

Figure 5

AACR American Association for Cancer Research

Clinical Cancer Research

Pharmacological inhibition of NOS activates ASK1/JNK pathway augmenting docetaxel-mediated apoptosis in triple negative breast cancer

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