

Breast Cancer Research and Treatment

Smac Mimetic Birinapant Induces Apoptosis and Enhances TRAIL Potency in Inflammatory Breast Cancer Cells in an IAP-Dependent and TNF- α -Independent Mechanism

--Manuscript Draft--

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Abstract:	<p>X-linked Inhibitor of Apoptosis Protein (XIAP), the most potent mammalian caspase inhibitor, has been associated with acquired therapeutic resistance in inflammatory breast cancer (IBC), an aggressive subset of breast cancer with an extremely poor survival rate. The second mitochondria-derived activator of caspases (Smac) protein is a potent antagonist of IAP proteins and the basis for the development of Smac mimetic drugs. Here, we report for the first time that bivalent Smac mimetic Birinapant induces cell death as a single agent in TRAIL-insensitive SUM190 (ErbB2-overexpressing) cells and significantly increases potency of TRAIL-induced apoptosis in TRAIL-sensitive SUM149 (triple negative, EGFR-activated) cells, two patient tumor-derived IBC models. Birinapant has high binding affinity (nM range) for cIAP1/2 and XIAP. Using isogenic SUM149- and SUM190-derived cells with differential XIAP expression (SUM149 wtXIAP, SUM190 shXIAP) and another bivalent Smac mimetic (GT13402) with high cIAP1/2 but low XIAP binding affinity ($K_d > 1 \mu\text{M}$), we show that XIAP inhibition is necessary for increasing TRAIL potency. In contrast, single agent efficacy of Birinapant is due to pan-IAP antagonism. Birinapant caused rapid cIAP1 degradation, caspase activation, PARP cleavage, and NF-κB activation. A modest increase in TNF-α production was seen in SUM190 cells following Birinapant treatment, but no increase occurred in SUM149 cells. Exogenous TNF-α addition did not increase Birinapant efficacy. Neutralizing antibodies against TNF-α or TNFR1 knockdown did not reverse cell death. However, pan-caspase inhibitor Q-VD-OPh reversed Birinapant-mediated cell death. In addition, Birinapant in combination or as a single agent decreased colony formation and anchorage-independent growth potential of IBC cells. By demonstrating that Birinapant primes cancer cells for death in an IAP-</p>

dependent manner, these findings support the development of Smac mimetics for IBC treatment.

Response to Reviewers:

November 15, 2012

Dr. Marc E. Lippman

Editor-in-Chief

Breast Cancer Research and Treatment

Re: Revised Manuscript (BREA-D-12-08576)

Title: Smac Mimetic Birinapant Induces Apoptosis and Enhances TRAIL Potency in Inflammatory Breast Cancer Cells in an IAP-Dependent and TNF- α -Independent Mechanism

Dear Dr. Lippman,

The above manuscript was initially submitted to Breast Cancer Research and Treatment and the authors received the reviews along with the decision of 'could be accepted for publication should you be prepared to incorporate major revisions'.

We thank the reviewer for his recommendations that were indeed helpful in revising this manuscript. We have included a detailed author response and revision status of each of the reviewer's comments.

In response to the reviewer's concerns, we have undertaken the following experiments:

- 1.Measurement of clonogenic growth potential as a means of assessing viability effects of the Smac mimetic treatments
- 2.Annexin-V/7-AAD flow cytometric staining of cells treated with the Smac mimetics as a direct means of evaluating apoptosis
- 3.An in-depth time course analysis of NF-kB activation in SUM190 cells following treatment with the Smac mimetic
- 4.Assessment of anchorage independent growth potential as a preliminary predictor of in vivo activity of the Smac mimetic

We sincerely hope that all the reviewers' concerns have been adequately addressed and the manuscript is acceptable for publication in the present revised form.

Disclosure Statement: The authors are aware of and agree to the content of the paper and their being listed as an author in the manuscript. We authorize the release of the data and this manuscript has not been submitted to any other journal.

As per the instructions, we have submitted the manuscript online. We thank you for your time and assistance.

Sincerely,

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Authors' Response to Reviewers Comments

We thank the reviewers for their recommendations that were indeed helpful in revising this manuscript. Please find below detailed response of the authors to the comments.

Critique 1: There is a significant issue with the use of trypan blue exclusion as the sole marker of determining the response of cell lines to drugs being evaluated. This is an outdated method of cell viability that does not directly relate to cell survival. There are multiple alternatives available that could be used as a more specific marker of the status of cells following drug treatment. To demonstrate the therapeutic relevance of the Smac mimetics in this manuscript, as an example, the trypan blue exclusion assay should be either replaced or complemented with another assay such as clonogenic growth, which is a more suitable measure of survival of cells following exposure to

therapeutic agents.

Author Response: As per the reviewer's suggestion, we have utilized a clonogenic growth assay to complement and confirm our previously presented findings, which were conducted using trypan blue viability assay. These new data have been included in parallel with trypan blue throughout the manuscript in Figures 3A, 3B, 3C, 5C, 7B, and 7D, and are very consistent with the findings presented prior to review.

Critique 2: Despite the title of the manuscript, there are no studies directly evaluating the effects of Birinapant on apoptosis detected by TUNEL, DNA fragmentation, or any other apoptosis-related parameter. Direct analysis of apoptosis in response to Birinapant and GT1402 would add strength to the manuscript.

Author Response: In the original submission, data was included showing both caspase activation (Figure 1C) and PARP cleavage (Figure 1C and 2E), which are established apoptotic parameters, and reversal of Birinapant-induced cell death by a caspase inhibitor (Q-VD-OPh), further supporting a caspase-dependent apoptotic mechanism of cell death. In response to the reviewer's suggestion, we have now included Annexin-V/7-AAD flow cytometric staining in the revised manuscript as a direct measurement of apoptosis since Annexin-V staining is specific to the phosphatidyl-serine (PS) residues on the cell surface that are translocated from the inner to the outer leaflet of the plasma membrane specifically during early apoptosis. The new data is now presented in Figures 1D and 2D for SUM149 and SUM190 cells respectively.

Critique 3: Given that one of the first characteristics of IBC to be identified was activation of NF-kB, the fact that Birinapant increased NF-kB phosphorylation in SUM190 cells undergoing apoptosis may signal that this therapeutic approach may not be relevant to effective treatment of IBC tumors in the in vivo setting. A demonstration of an in vivo effect would increase the impact of the claims in this manuscript that Smac mimetics could be successfully developed for IBC therapy.

Author Response: The authors understand the concerns for in vivo relevance raised by the reviewer, and have undertaken two sets of experiments to address those concerns.

First, the authors conducted a time course analysis of NF-kB activation in SUM190 cells following treatment with Birinapant and found that NF-kB activation was transient. While NF-kB activation was observed 4 h after treatment, NF-kB phosphorylation levels and total protein were reduced at 8 and 12 h, and had recovered to levels comparable to the untreated sample after 24 h. This data can now be found in Figure 2F. It is further supported by the fact that Birinapant does not induce TNF- α production in the IBC cells (a primary cause for the paradoxical NF-kB activation seen in some cancer models). We have also addressed this in the discussion section, comparing and contrasting other studies related to NFkB activation mediated by TNF- α .

Secondly, the authors conducted an anchorage-independent growth assay in both SUM149 and SUM190 cells. Anchorage-independent growth is considered a surrogate model for tumorigenicity, and is often an effective predictor of in vivo treatment efficacy. Following treatment with Birinapant (alone in SUM190 or in combination with TRAIL in SUM149), the potential for anchorage-independent growth was significantly reduced. This data has been incorporated into the manuscript in Figure 4A and 4B. We believe that because the activation of NF-kB survival signaling following treatment is only transient, combined with the fact that cells underwent apoptosis and exhibited a decrease in clonogenicity and anchorage-independent cell growth in response to Birinapant, this treatment plan has potential to be efficacious in vivo.

This manuscript is the first study in IBC models with detailed characterization of the IAP binding affinity kinetics and mechanism of the two Smac mimetic compounds and reveals both single agent efficacy and combinatorial function with TRAIL. In vivo studies in IBC murine tumor models are planned in the near future, as we are currently conducting pilot studies regarding dosing, route, and schedule of treatment with the

Smac mimetics.

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4 **Smac Mimetic Birinapant Induces Apoptosis and Enhances TRAIL Potency in Inflammatory**
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6 **Breast Cancer Cells in an IAP-Dependent and TNF- α -Independent Mechanism**
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4 **Abstract**
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6 X-linked Inhibitor of Apoptosis Protein (XIAP), the most potent mammalian caspase inhibitor, has been
7 associated with acquired therapeutic resistance in inflammatory breast cancer (IBC), an aggressive subset
8 of breast cancer with an extremely poor survival rate. The second mitochondria-derived activator of
9 caspases (Smac) protein is a potent antagonist of IAP proteins and the basis for the development of Smac
10 mimetic drugs. Here, we report for the first time that bivalent Smac mimetic Birinapant induces cell death
11 as a single agent in TRAIL-insensitive SUM190 (ErbB2-overexpressing) cells and significantly increases
12 potency of TRAIL-induced apoptosis in TRAIL-sensitive SUM149 (triple-negative, EGFR-activated)
13 cells, two patient tumor-derived IBC models. Birinapant has high binding affinity (nM range) for cIAP1/2
14 and XIAP. Using isogenic SUM149- and SUM190-derived cells with differential XIAP expression
15 (SUM149 wtXIAP, SUM190 shXIAP) and another bivalent Smac mimetic (GT13402) with high cIAP1/2
16 but low XIAP binding affinity ($K_d >1 \mu\text{M}$), we show that XIAP inhibition is necessary for increasing
17 TRAIL potency. In contrast, single agent efficacy of Birinapant is due to pan-IAP antagonism. Birinapant
18 caused rapid cIAP1 degradation, caspase activation, PARP cleavage, and NF- κ B activation. A modest
19 increase in TNF- α production was seen in SUM190 cells following Birinapant treatment, but no increase
20 occurred in SUM149 cells. Exogenous TNF- α addition did not increase Birinapant efficacy. Neutralizing
21 antibodies against TNF- α or TNFR1 knockdown did not reverse cell death. However, pan-caspase
22 inhibitor Q-VD-OPh reversed Birinapant-mediated cell death. In addition, Birinapant in combination or as
23 a single agent decreased colony formation and anchorage-independent growth potential of IBC cells. By
24 demonstrating that Birinapant primes cancer cells for death in an IAP-dependent manner, these findings
25 support the development of Smac mimetics for IBC treatment.
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53 **Keywords:** Smac/DIABLO, inhibitor of apoptosis protein (IAP), TRAIL, inflammatory breast cancer
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4 **Introduction**
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6 Acquired therapeutic resistance due to tumor cell adaptation to persistent therapeutic stress is an
7 unmet challenge in inflammatory breast cancer (IBC), one of the most lethal subtypes of breast cancer [1].
8 We have identified XIAP overexpression via post-transcriptional mechanisms as a dominant feature of
9 apoptotic dysregulation involved in acquired therapeutic resistance to epidermal growth factor receptor
10 (EGFR) and/or ErbB2/HER2-targeting drugs like trastuzumab and lapatinib as well as apoptosis inducing
11 agents such as TRAIL (TNF-related apoptosis inducing ligand) [2-5]. This is supported by other studies
12 that have shown consistent differences in molecular phenotype between IBC and locally advanced non-
13 IBC tumors, with IBC characterized by a hyperproliferative state and apoptotic dysregulation [2-4, 6-13].
14 Thus, the development of novel therapeutic strategies targeting the anti-apoptotic signaling pathway is
15 highly relevant in IBC.
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29 XIAP, the most potent caspase inhibitor, is a member of the highly conserved inhibitor of apoptosis
30 protein (IAP) family of endogenous apoptosis inhibitors. IAPs have been shown to inhibit apoptosis
31 induced by both extrinsic (e.g., death receptor) and intrinsic (e.g., DNA damage) stimuli. They are
32 characterized by the presence of 1-3 baculoviral IAP repeat (BIR) domains which mediate interactions
33 with other proteins. Cellular IAP 1 and 2 (cIAP1 and 2) were originally identified as components of the
34 TNF- α receptor (TNFR2) complex and are increasingly recognized as modulators of diverse extrinsic
35 signals. They possess a C-terminal RING (Really Interesting New Gene) domain which has E3 ubiquitin
36 ligase activity. By maintaining the ubiquitination states of key substrates such as RIP kinases and
37 caspases, they are able to promote survival signaling. XIAP, in addition to harboring a C-terminal RING
38 domain with E3 ligase activity, is a direct caspase inhibitor which can bind to activated caspases-3, -7,
39 and -9 and inhibit their proteolytic activity [14].
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53 While XIAP is not considered to be a classical oncogene, it has been detected at elevated levels in
54 tumors resistant to chemotherapy and is associated with poor outcome in several cancers [15-18]. Two
55 broad approaches have been taken to develop clinical inhibitors of XIAP, including antisense
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4 oligonucleotides that reduce XIAP expression and small molecule inhibitors that antagonize XIAP's
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6 caspase inhibitory function. These molecules are currently being evaluated in preclinical and clinical
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8 phase I-II studies [19, 20]. One class of XIAP inhibitory molecules being developed as anticancer
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10 therapeutic agents is Smac mimetics, which are modeled after the AVPI tetrapeptide IAP binding motif
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12 (IBM) of Smac/DIABLO [21]. Smac mimetics are attractive since Smac/DIABLO is considered to be one
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14 of the main antagonists of XIAP and other IAPs because it can bind to the BIR3 region of IAP proteins
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16 through its *N*-terminal AVPI tetrapeptide. In addition, XIAP overexpression can inhibit the release of
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18 endogenous Smac from the mitochondria [22]. Recently, expression of Smac/DIABLO in 62 breast
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20 cancer patient specimens assessed by flow cytometry was shown to inversely correlate with tumor stage
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22 [23]. Smac mimetics are potent pro-apoptotic agents, and their mechanisms include: 1. sterically and/or
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24 competitively occluding the binding sites of XIAP from caspases-3, -7 and -9; 2. XIAP degradation; 3.
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26 TNF- α -mediated cell death; and 4. inhibition and degradation of other IAP family members. This is
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28 important due to the fact that in many cases when XIAP is targeted, other IAPs suppress apoptosis by
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30 compensatory mechanisms [24-28]. Currently, Smac mimetics including Birinapant (formerly called
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32 TL32711) are in clinical trials [29]. While only a subset of tumor cell lines is sensitive to Smac mimetics
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34 as single agents, combining Smac mimetics with different therapeutic agents has been shown to increase
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36 potency of those agents [30-35]. The predominant mechanism of many of the Smac mimetic agents seems
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38 to be TNF- α dependent [28, 36]. Here we demonstrate that the mechanism of action of Birinapant as a
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40 single agent and in combination with TRAIL is not dependent on TNF- α , but is predominantly due to IAP
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42 downregulation and caspase activation in the inflammatory breast cancer cellular models.
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Materials and Methods

Cell Lines and Reagents—SUM149 and SUM190 IBC cell lines were obtained from Asterand, Inc. (Detroit, MI) and cultured as described previously [3]. Asterand characterizes cell lines using short tandem repeat polymorphism analysis. Cells were banked upon receipt and cultured for no more than 6 months prior to use in this study. SUM149 cells stably expressing wtXIAP and FG9 vector control were generated and maintained as described previously [4]. TRAIL was purchased from Enzo Life Sciences (Farmingdale, NY), and TNF- α was purchased from Sigma Aldrich (St. Louis, MO). A TNF- α neutralizing antibody was purchased from R&D Systems (Minneapolis, MN), and Q-VD-OPh pan-caspase inhibitor was purchased from Cal Biochem (Billerica, MA). TNFR1 siRNA, control scramble siRNA (Thermo/Dharmacon On-Target plus SMART pool reagents: Control siRNA #L-004445-00-0005, TNFRSF1A #L-005197-00-0005), and Dharmafect transfection reagent were purchased from Thermo Scientific. Smac mimetics Birinapant and GT13402 were obtained from TetraLogic Pharmaceuticals (Malvern, PA) and have been designed based on the four *N*-terminal amino acids of Smac/DIABLO (Ala-Val-Pro-Ile) and caspase-9 (Ala-Thr-Pro-Phe) [29].

Generation of XIAP-knockdown IBC cell line—SUM190 cells stably expressing shRNA against XIAP and FG12 vector control were generated using a lentiviral expression system (kindly provided by Dr. Colin Duckett, University of Michigan, Ann Arbor, MI). Briefly, HEK293T cells were transfected using polyethylenimine with 5 μ g of pHCMV, pRRE, and pRSVrev [37], which drive the expression of lentiviral structural proteins, and 5 μ g of pFG12 shXIAP GFP or pFG12 GFP DNA; 24 h post-transfection, media was changed. After 40 h post-transfection, the virus-containing media was collected from the HEK293T cells and filtered through a 0.45 μ m Millex HV PVDF filter unit (Millipore, Billerica, MA) onto cells with 25 mM polybrene (Sigma Aldrich). After four hours, fresh media was added, and cells were incubated for an additional 48 h at 37°C, 5% CO₂. Stable shXIAP and FG12 cells were sorted using FACS for high GFP expression.

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7 *Fluorescence Polarization Assay*—The binding affinities of compounds to XIAP and cIAP1 were
8 determined as described previously [38] using a fluorogenic substrate and are reported as K_d values.
9 Initially, the dissociation constant (K_d) for the fluorescently labeled modified Smac peptide (AbuRPF-
10 K(5-Fam)-NH₂; FP peptide) was determined by using a fixed concentration of peptide (5 nM) and
11 titrating varying concentrations of protein (0.075 μ M – 5 μ M in half log dilutions). The dose-response
12 curves were produced by a non-linear least squares fit to a single-site binding model using GraphPad
13 Prism (Graphpad Software, La Jolla, CA; data not shown), with 5 nM of FP peptide and 50 nM of XIAP
14 used in the assay. Various concentrations of Smac mimetics (100 μ M – 0.001 μ M in half log dilutions)
15 were added to FP peptide:protein binary complex for 15 minutes at room temperature in 100 μ L of 0.1 M
16 potassium phosphate buffer, pH 7.5, containing 100 mg/mL bovine γ -globulin. Following incubation, the
17 polarization values were measured on a Perkin-Elmer Victor²V multi-label plate reader using a 485 nm
18 excitation filter and a 520 nm emission filter. IC₅₀ values were determined from the plot using non-linear
19 least squares analysis in Graphpad Prism. Calculations were based on a maximum signal (protein BIR:FP
20 peptide complex treated with DMSO alone) after subtraction of background.
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40 *Immunoblot Analysis*—Western immunoblot analysis was carried out as described previously [2]. Cells
41 were harvested at 4 h or 24 h post treatment. Membranes were incubated with primary antibodies for
42 XIAP (BD Biosciences, San Jose, CA, 1:1000), DR4, actin, GAPDH, and PARP (Santa Cruz
43 Biotechnology, Santa Cruz, CA, 1:4000), cIAP1 and cIAP2 (R&D Systems, 1:1000), caspase-8, JNK, p-
44 JNK, NF- κ B (p65), p-NF- κ B (p65), TNFR1 and caspase-3 (Cell Signaling Technologies, Danvers, MA,
45 1:1000) at 4°C overnight. Membranes were washed and incubated with anti-mouse or anti-rabbit HRP-
46 conjugated antibodies (Cell Signaling Technologies) for 1 h at room temperature. Chemiluminescent
47 substrate (Thermo Scientific, Waltham, MA) was applied for 5 min, and membranes were exposed to
48 film. Densitometric analysis was performed using NIH ImageJ software [39].
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7 *Cell Viability*—Trypan blue exclusion assay was performed as described previously [2]. Cells were
8 seeded in 6 well plates at 7.5×10^4 (SUM149) or 1.5×10^5 (SUM190) cells per well and allowed to adhere
9 overnight. Cells were treated with TRAIL (0-100 ng mL⁻¹), Birinapant (0-10,000 nM), GT13402 (0-
10 10,000 nM), TNF- α (50 ng mL⁻¹), TNF- α neutralizing antibody (10 μ g mL⁻¹), pan-caspase inhibitor Q-
11 VD-Oph (20 μ M), or a combination as indicated. All treatments were applied for 24 h, then cells were
12 trypsinized and resuspended in DPBS. Next, 10 μ L of cell suspension was added to 10 μ L 0.4% trypan
13 blue, and 10 μ L of the mixture was loaded onto a hemocytometer; cells were counted, and live and dead
14 cell numbers were recorded.
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26 *Clonogenic Growth Assay*- Cells were plated in triplicate in 6 well plates at 250-500 cells/well (SUM149)
27 or 500-1000 cells/well (SUM190) and allowed to adhere overnight. Cells were treated with TRAIL (0-
28 100 ng mL⁻¹), Birinapant (0-10,000 nM), GT13402 (0-10,000 nM), TNF- α (50 ng mL⁻¹), TNF- α
29 neutralizing antibody (10 μ g mL⁻¹), pan-caspase inhibitor Q-VD-Oph (20 μ M), or a combination as
30 indicated. After 24 h treatments, the cells were washed twice with PBS, and regular growth media was
31 added. The cells were then allowed to grow for 5-14 days, changing the media every 4-5 days. Once
32 colonies of at least 50 cells were observed, the cells were washed with PBS, fixed, stained with 0.4%
33 crystal violet, then rinsed in cold water and left to dry overnight. Colonies were counted and imaged using
34 a ColCount (Oxford Optronix, Oxford, UK), and colonies formed per cells plated was calculated.
35 Numbers were normalized to untreated.
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51 *Anchorage Independent Growth Assay*- Cells were plated in 6 well plates at 7.5×10^4 (SUM149) or
52 1.5×10^5 (SUM190) cells/well and allowed to adhere overnight. Treatments were applied for 24 h, after
53 which cells were harvested and counted with trypan blue viability stain. A base layer of 0.7% agarose in
54 regular growth medium was poured into wells of a 12 well plate and allowed to solidify at 4°C. Then
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4 1.25x10⁴ cells/well from each treatment were plated in triplicate in 0.45% agarose in regular growth
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6 medium on top of the base layer and allowed to solidify at 4°C. Plates were then transferred to a 37°C
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8 incubator with 5% CO₂ and allowed to grow for 14 to 21 days. Once visible colonies had formed, they
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10 were counted under a microscope, and colony counts were normalized to the untreated sample. Images of
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12 representative fields were taken with 5x magnification using a Zeiss Axio Observer A1 microscope
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14 (Thornwood, NY), Hamamatsu Orca ER digital camera (Bridgewater, NJ), and MetaMorph software
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16 (Molecular Devices, Sunnyvale, CA).
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22 *Annexin-V Staining*- Cells were seeded in 6 well plates at 7.5x10⁴ (SUM149) or 1.5x10⁵ (SUM190) cells
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24 per well and allowed to adhere overnight. Cells were treated with TRAIL (0-100 ng mL⁻¹), Birinapant (0-
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26 1000 nM), GT13402 (0-1000 nM), or a combination as indicated for 12 h. Cells were harvested with
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28 0.25% trypsin (- EDTA), washed with PBS, and resuspended in biotin-conjugated Annexin-V (Beckman
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30 Coulter, Brea, CA) for 5 min at RT. Cells were washed again with PBS and resuspended in streptavidin-
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32 conjugated FITC (Life Technologies, Grand Island, NY) and 7-AAD (BD Pharminogen, San Jose, CA)
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34 dyes for 15 min on ice. Cells were washed and resuspended in PBS, and then at least 25,000 events were
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36 collected on a BD FACSCalibur flow cytometer. Results were analyzed using FlowJo software (Tree Star,
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38 Inc., Ashland, OR).
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44 *TNF-α Measurement*—TNF-α protein levels were measured in cell culture supernatants using the BD
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46 OptEIA TNF-α ELISA kit II according to the manufacturer's instructions. Briefly, 200 μL of culture
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48 supernatants were tested in triplicate. A standard curve was generated using the manufacturer supplied
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50 standard. Absorbance was read at 450 nm on a Perkin-Elmer Victor²V multi-label plate reader (Waltham,
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52 MA). The 570 nm background correction was subtracted from 450 nm values to yield the final results.
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TNFR1 Knockdown—Cells were seeded in 6 well plates at 1.5×10^5 cells per well and allowed to adhere overnight. After 24 h, either scramble control siRNA or TNFR1 targeting siRNA at 100 nM was applied in the presence of Dharmafect transfection reagent. Birinapant (0-1000 nM) was added the day after transfection, and cells were harvested after 24 h for trypan blue viability staining and western immunoblotting to confirm knockdown.

Statistical Analysis—Statistical analyses were performed using Graphpad Prism Version 4 and the student's two-tailed t-test. Differences were considered significant at $p < 0.05$.

Results

IAP Binding Affinity of Novel Bivalent Smac Mimetics—A fluorescence polarization assay was used to determine the dissociation constants (K_d) of Smac mimetics Birinapant and GT13402 for XIAP and cIAP1 by monitoring the decrease in fluorescence polarization signal due to competitive displacement of an FP peptide from the BIR3/FP peptide binary complex. Both Smac mimetic compounds induced a dose-dependent decrease in the fluorescence signal. As shown in Table 1, the binding constants (K_d) of Birinapant for XIAP and cIAP1 were determined to be 45 nM and <1nM respectively. In contrast, GT13402 had lower affinity for XIAP with the K_d close to 1 μ M but similar affinity for cIAP1 as Birinapant (K_d <1 nM)

Smac Mimetic Enhances TRAIL Potency in a TRAIL-Sensitive IBC Cell Line by cIAP1/2 Degradation and Caspase-8 and PARP Cleavage—The basal-type IBC cell line SUM149 has been observed to be TRAIL-sensitive [40, 41], which was supported by data in Figure 1A displaying a dose-dependent decrease in viability following TRAIL treatment (0-100 ng mL⁻¹). Characterization of the Smac mimetic compounds as single agents in SUM149 cells revealed that neither Birinapant nor GT13402 is effective in inducing cell death as measured by trypan blue exclusion assay, with approximately 95% of cells still viable following treatment with the highest concentrations tested (1000 nM Birinapant, 10,000 nM GT13402- Fig. 1A). However, combinations of TRAIL (10-50 ng mL⁻¹) and Birinapant (0-1000 nM) enhanced TRAIL potency in SUM149 cells, with a statistically significant ~25% reduction in viability achieved with as low as 25 ng mL⁻¹ TRAIL + 30 nM Birinapant (Fig. 1B). GT13402, which has low affinity for XIAP compared to Birinapant, did not significantly enhance TRAIL potency, even at the highest concentration (10,000 nM) tested (Fig. 1B). Birinapant as a single agent caused significant degradation of cIAP1 and 2, which was not enhanced by addition of TRAIL (Fig. 1C). While a slight decrease in XIAP expression can be seen following TRAIL treatment, no significant decrease in XIAP levels was observed following combinatorial treatments (Fig. 1C). However, Birinapant+TRAIL-treated cells showed increased levels of active caspase-8 and caspase-3, as well as PARP cleavage, over single agents within 4

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4 h of treatment, indicative of apoptosis-mediated cell death (Fig. 1C). Annexin-V/7-AAD flow cytometric
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6 staining further confirmed the mechanism of death to be apoptosis, with a dose-dependent increase in
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8 cells staining positive for Annexin-V (white+gray bar) from approximately 30% with TRAIL alone to 50-
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10 55% following treatment with Birinapant+TRAIL after 12 h (Fig. 1D).

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14 Because XIAP overexpression has been identified as a critical factor in TRAIL resistance [15, 42,
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16 43], we wanted to delineate the role of XIAP expression in sensitivity to Smac mimetics. To do this, we
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18 characterized both Birinapant and GT13402, which have differential affinity for XIAP, in SUM149
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20 wtXIAP, a SUM149-derived isogenic cell line with stable exogenous XIAP overexpression [4]. Data in
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22 Figure 1E show that Birinapant was more effective in increasing TRAIL potency than GT13402 in
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24 SUM149 wtXIAP. This reveals the IAP specificity of the Smac mimetics and the importance of targeting
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26 XIAP along with cIAP1 and 2 in this model.
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33 *Smac Mimetics Induce Apoptosis as Single Agents in a Dose-Dependent Manner in TRAIL-Resistant*
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35 *SUM190 IBC Cells*—In order to evaluate the efficacy of the Smac mimetics in a TRAIL-resistant cell
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37 line, we characterized Birinapant and GT13402 in SUM190, a cell line isolated from a patient IBC tumor
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39 with ErbB2-overexpression and insignificant DR4 expression (a factor that can contribute to TRAIL
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41 resistance) as shown in Figure 2A. SUM190 cells were only moderately sensitized to TRAIL (Fig. 2B,
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43 graph) when XIAP was stably downregulated using an XIAP-targeting shRNA lentiviral construct
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45 (SUM190 shXIAP- Fig. 2B, immunoblot).
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48 In contrast, Birinapant, which targets cIAP1/2 and XIAP with high affinity, significantly
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50 decreased the viability of SUM190 cells in a dose-dependent manner as a single agent. Data in Figure
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52 2C, top panel show approximately 50% cell death at 300 nM as measured by trypan blue exclusion assay.
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54 The requirement of pan-IAP antagonism is supported by the observations (Fig. 2C): 1. treatment with
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56 GT13402 (lower affinity for XIAP) was also effective as a single agent in inhibiting SUM190 cell
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4 viability; 2. Birinapant (high affinity for cIAP1/2 and XIAP) is more potent (20% viability at 1000 nM)
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6 than GT13402 (60% viability at 1000 nM) in this viability assay; 3. Birinapant treatment in the XIAP
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8 knockdown cell line (SUM190 shXIAP) caused an overall reduction in viability at lower doses (30-
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10 300nM) compared to the parental SUM190 cells at the corresponding doses; 4. the difference in potency
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12 between the two compounds with differential affinity for XIAP was attenuated in the XIAP-knock down
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14 cell line (Fig. 2C, lower panel).
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17 To confirm apoptosis with Birinapant treatment, immunoblot analysis was conducted and data in
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19 Figure 2D showed a significant decrease in cIAP1 levels and enhanced PARP cleavage. However, no
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21 decrease in XIAP expression or JNK phosphorylation levels was observed (Fig. 2D). In addition,
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23 Annexin-V/7-AAD staining by flow cytometric analysis was carried out at an early time point (12 h).
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25 Data in Figure 2E show an increase in Annexin-V positive cells (white+gray bar) with no change in 7-
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27 AAD single positive cells (black bar), further supporting an apoptotic mechanism of cell death.
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30 Because pro-survival NF- κ B signaling can interfere with cell death, we conducted a time course study to
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32 evaluate its activation post-Birinapant treatment. Data in Figure 2F show that there is an initial increase in
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34 p-NF κ B at 4 h with no change in total protein levels. However, at 8-12 h time points, a time-dependent
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36 decrease in both total and phosphorylated NF- κ B is observed. By 24 h, expression of both total and
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38 phosphorylated protein returns to untreated levels. Together, these results indicate the single agent
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40 efficacy of Smac mimetics in the SUM190 IBC cellular model.
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46 *Smac Mimetics Inhibit Clonogenic Growth Capacity in Combination with TRAIL and as Single Agents in*
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48 *SUM149 and SUM190 Cells Respectively*—To determine the effect of Smac mimetics on the growth
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50 characteristics of IBC cells, clonogenic growth potential was assessed. Data in Figure 3A show that in
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52 TRAIL-sensitive SUM149 cells, Birinapant+TRAIL induces a significant reduction in clonogenic
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54 potential even at lower doses (40% decrease in colony formation at 30 nM Birinapant +10 ng mL⁻¹
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56 TRAIL compared to 10 ng mL⁻¹ TRAIL). Representative images show the decrease in the number of
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4 colonies formed with Birinapant+TRAIL vs. TRAIL alone. Further, Birinapant was more potent than
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6 GT13402 in increasing TRAIL efficacy, with only 8% colony formation efficiency at 100 nM Birinapant
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8 + 50 ng mL⁻¹ TRAIL, compared to 32% colony formation efficiency with 100 nM GT13402 + 50 ng mL⁻¹
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10 TRAIL (Fig. 3B). Similar to our results from the trypan blue exclusion assay in Figure 1, neither
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12 Birinapant nor GT13402 caused any change in SUM149 colony formation compared to untreated cells as
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14 single agents (data not shown).
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19 Unlike in SUM149 cells, both Smac mimetics were similarly effective at inhibiting the
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21 clonogenic growth potential of TRAIL-resistant SUM190 as single agents (Fig. 3C). Representative
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23 images show a decrease in SUM190 colony formation following treatment with Birinapant and GT13402.
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29 *Birinapant Inhibits Anchorage-Independent Growth (AIG) Potential*—Anchorage-independent colony
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31 formation in soft agar has been widely used as a surrogate *in vitro* model to assess cancer cell tumorigenic
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33 potential and is considered to be a reasonably good predictor of *in vivo* activity [44, 45]. Data in Figure
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35 4A reveal that Birinapant+TRAIL (black bar) causes a 55% reduction in the number of colonies formed
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37 while TRAIL alone (white bar) causes a 20% decrease relative to untreated. Representative images of
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39 colonies formed in soft agar are shown in the right panel of Figure 4A, showing similar sized colonies in
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41 all treatment groups but a significant reduction in colony number of the Birinapant+TRAIL combination.
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43 Analysis of AIG in SUM190 cells, wherein we observed single agent efficacy of Birinapant, a dose-
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45 dependent decrease in AIG following treatment with Birinapant (100-10,000 nM) is observed (Fig. 4B).
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47 Data show a significant ~50% decrease in the number of colonies formed with 100 nM Birinapant
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49 treatment relative to untreated (Fig. 4B). Representative images of colonies formed in soft agar are shown
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51 in the right panel of Figure 4B, with no observable change in colony size, but a noticeable reduction in
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53 colony number with increasing dose of Birinapant.
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4 *Smac Mimetic Efficacy in SUM149 and SUM190 Cells is TNF- α -Independent and Caspase-Dependent—*

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6 Studies were then conducted to evaluate whether TNF- α influences Smac mimetic efficacy in IBC
7 cellular models. Since Smac mimetics, in addition to binding IAPs, have been shown to induce TNF- α
8 production in sensitive cells, we measured TNF- α levels in the conditioned media of Birinapant-treated
9 SUM149 and SUM190 cells by ELISA. Data in Figure 5A reveal insignificant TNF- α secretion in
10 SUM149. However, SUM190 cells, which are sensitive to Smac mimetics as single agents, produce
11 autocrine TNF- α in a dose-dependent fashion in response to Birinapant treatment, although it should be
12 noted that the levels were in the low picogram mL⁻¹ range. Addition of exogenous TNF- α (50 ng mL⁻¹) in
13 combination with Birinapant in SUM190 cells had no significant additive effect on cell death compared to
14 Birinapant alone as measured by trypan blue exclusion (Fig. 5B) and assessment of clonogenic growth
15 capacity (Fig. 5C).
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29 Next, the effect of TNF- α receptor (TNFR1) knockdown was evaluated in SUM190 cells using
30 TNFR1-targeting siRNA and control scrambled siRNA at 24 h. Birinapant-induced cell death was not
31 reversed (Fig. 6A) by specific TNFR1 downregulation (Fig. 6B). Further, addition of a TNF- α
32 neutralizing antibody (10 μ g mL⁻¹) as in a method described previously [46] to SUM149 (Fig. 7A,B) or
33 SUM190 (Fig. 7C,D) cells did not reverse the decrease in viability or colony formation efficacy of
34 Birinapant in combination or as a single agent.
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43 In contrast to the TNF- α studies above, the apoptosis-inducing effect of Birinapant+TRAIL in
44 SUM149 is potently reversed in the presence of pan-caspase inhibitor Q-VD-OPh, as evidenced by trypan
45 blue exclusion assay and clonogenic growth assay (Fig. 7A,B). Similar reversal using Q-VD-OPh is
46 observed in Birinapant-treated SUM190 cells (Fig. 7C,D). Together, these results reveal a TNF- α -
47 independent but IAP- and caspase-dependent mechanism of action of Birinapant +TRAIL in SUM149
48 cells and Birinapant as a single agent in SUM190 cells.
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Discussion

We report herein for the first time, IAP binding affinity of Birinapant, a bivalent Smac mimetic, and its efficacy in inducing apoptosis in TRAIL-sensitive and TRAIL-resistant IBC cellular models with differential XIAP expression. In the TRAIL-sensitive basal-type SUM149 cell line, the Smac mimetic Birinapant was not effective as a single agent, but significantly enhanced TRAIL potency in a dose-dependent manner. In contrast, in the TRAIL-resistant, ErbB2-overexpressing SUM190 cell line, Birinapant significantly decreased cell viability and clonogenic growth potential as a single agent. Additionally, the effect of Birinapant on these cellular models was further characterized using anchorage-independent growth as a predictor of *in vivo* behavior. The ability of cells *in vitro* to grow in the absence of anchorage has been shown to correlate directly with the ability of those cells to cause tumors in multiple mouse models [44, 47]. Thus, the inhibition of anchorage-independent growth seen in Birinapant-treated SUM190 cells and Birinapant+TRAIL-treated SUM149 cells highlights Birinapant's ability to target tumorigenic cells and further supports Birinapant's potential for efficacy *in vivo*.

IBC is a particularly aggressive subset of breast cancer that is characterized by rapid progression, local and distant metastasis, younger age of onset, and less than 5% survival rate beyond 5 years when treated with surgery or radiation therapy [1]. Apoptotic dysregulation has been observed in IBC cells and in patient tumors [2-4, 8, 9, 12, 13]. We have previously generated isogenic IBC cell models that are derived from SUM149 and SUM190 cell lines, in which therapeutic resistance to the dual ErbB1/2 tyrosine kinase inhibitor lapatinib is exhibited [2-4]. One of the dominant features in the resistant cell lines was overexpression of XIAP, which correlated directly with therapeutic resistance. We also reported that XIAP overexpression was not due to increased mRNA or protein stability, but rather due to increased IRES-mediated cap-independent translation of XIAP mRNA in response to lapatinib treatment [4]. The resistant cell lines also exhibited cross-resistance to other agents like TRAIL [5]. Thus, it is of particular interest to identify inhibitors of XIAP, as well as other members of the IAP family, in order to prevent or reverse acquired therapeutic resistance in IBC. In doing so, it may be possible to treat IBC with an IAP

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4 inhibitor alone, or to sensitize these tumors to other drugs as part of a combination regimen. One way to
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6 inhibit the IAP family of proteins is through the use of Smac mimetics, which act in a manner similar to
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8 Smac/DIABLO, a known effector of IAP degradation in apoptosis initiation.
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11 In the present study, we characterized parental SUM149 and SUM190 IBC cell lines and their
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13 isogenic derivatives with differential XIAP expression (SUM149 wtXIAP, SUM190 shXIAP) for Smac
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15 mimetic sensitivity. These multiple lines were treated with Birinapant (pan-IAP antagonist with high
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17 affinity for cIAP1/2 and XIAP) and GT13402, a Smac mimetic with lower affinity for XIAP. The
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19 differential binding of these two new Smac mimetics provides a small molecule approach to probe the
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21 impact of XIAP inhibition on Smac mimetic treatment in IBC. Both Birinapant and GT13402 caused
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23 degradation of cIAP1 and cIAP2 but had no effect on XIAP levels. In contrast, it has been recently
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25 reported that bivalent Smac mimetic BV6 degraded both cIAP1 and XIAP but not cIAP2 in glioblastoma
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27 cells, revealing differences in mechanism of action in different cell models [35]. In general, Smac
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29 mimetic compounds are more efficacious in enhancing potency of other chemotherapeutic agents [21]. In
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31 SUM149 cells, a similar phenomenon was observed as the cells were insensitive to Birinapant treatment
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33 alone, but the addition of Birinapant increased TRAIL efficacy.
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38 A significant finding was the single agent efficacy of Birinapant in the SUM190 cell line, which does
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40 not express death receptor DR4 and is insensitive to TRAIL treatment. Interestingly, previous studies
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42 have shown that Smac mimetics in certain cancer models can cause paradoxical activation of NF- κ B by
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44 autocrine TNF- α production and that the efficacy of Smac mimetics can be blunted by this activation of
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46 pro-survival NF- κ B signaling [27, 28, 36, 48-50], particularly in an *in vivo* setting. However, in the
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48 SUM190 IBC cell model, it appears that Birinapant efficacy may not be affected by NF- κ B activation
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50 since we observed transient changes, with slight activation and downregulation at earlier time points and
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52 activated and total NF- κ B returning to basal levels by 24 h. Further, this is supported by our observations
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54 that Birinapant efficacy is not TNF- α dependent. SUM190 cells showed only a modest dose-dependent
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56 increase in TNF- α levels at picogram mL⁻¹ quantities in Birinapant-treated culture supernatants. This is
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4 interesting since pro-apoptotic activity of other Smac mimetics is often associated with significant
5 autocrine production of TNF- α and interaction with death receptors [28, 36, 49]. A recent study in
6 pancreatic cells with another Smac mimetic reported that inhibition of TNF- α by a neutralizing antibody
7 reduced activation of caspases [50]. However, in the present study, addition of a neutralizing TNF- α
8 antibody or silencing of TNFR1 using siRNA failed to rescue SUM190 cells from Birinapant-induced cell
9 death. A similar phenomenon was observed in Birinapant+TRAIL treated SUM149 cells, where the
10 addition of a neutralizing TNF- α antibody failed to inhibit cell death. Furthermore, addition of exogenous
11 TNF- α did not enhance cell death in SUM190 after treatment with increasing doses of Birinapant,
12 supporting TNF- α -independent efficacy of Birinapant in the IBC cells.
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16 The present study shows Birinapant efficacy corresponded with significant cIAP1/2 degradation,
17 apoptotic parameters, and a reversal of Birinapant-induced cell death after addition of a caspase inhibitor.
18 A recent study has shown a monovalent Smac mimetic (LBW242) similarly induces cell death in a TNF-
19 α -independent, caspase-dependent manner in neuroblastoma cell lines [51]. In contrast to Birinapant,
20 LBW242 did not cause significant degradation of cIAP1/2. Further, the IC₅₀ values for LBW242 as a
21 single agent in the neuroblastoma cells lines were in the 50 μ M concentration range, causing significant
22 toxicity in matched normal cells. Birinapant, however is efficacious at lower nanomolar concentrations
23 tested herein and previously reported [29]. Together, these results indicate that the mechanism of action
24 of Birinapant in the IBC cell models tested is predominantly IAP-dependent, caspase-mediated apoptosis.
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28 Currently, there is no standard IBC-specific treatment plan for patients with advanced disease.
29 Birinapant is currently in a Phase 1a dose escalation study in patients with refractory solid tumors and
30 lymphoma [29]. By demonstrating that Birinapant primes IBC cells for death in an IAP-dependent
31 manner, this study strengthens the feasibility of developing Birinapant for IBC therapy.
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Ethical standards: This manuscript complies with the current laws of the United States of America.

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Table 1 Determination of binding constant (Kd) for XIAP by a fluorescence polarization assay

Compound	XIAP Kd (nM)	cIAP1 Kd (nM)
Birinapant	45	<1
GT13402	997	<1

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4 **Figure Legends**
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7 **Fig 1** *Smac mimetics sensitize SUM149 cells to TRAIL-mediated cell death.* **a**, Viability as determined by
8 trypan blue exclusion assay of SUM149 cells treated with Birinapant (0-1000 nM), GT13402 (0-10,000
9 nM), and TRAIL (0-100 ng mL⁻¹) alone. Bars represent mean±SEM viable cells taken as a percentage of
10 total cells (n=2-3; *P<0.05, #P<0.005, all comparisons made to untreated). **b**, Viability as determined by
11 trypan blue exclusion assay of SUM149 cells treated with TRAIL (10-50 ng mL⁻¹) alone or in
12 combination with Birinapant (0-1000 nM) or GT13402 (0-10,000 nM). Bars represent mean±SEM viable
13 cells taken as a percentage of total cells (n=3-5; *P<0.05, #P<0.005, all comparisons made between
14 TRAIL+Smac mimetic and same concentration of TRAIL alone). **c**, Western immunoblot analysis of
15 cIAP1, cIAP2, XIAP, caspase-3, caspase-8 and PARP expression in SUM149 cells treated with
16 Birinapant (0-1000 nM) ± TRAIL (50 ng mL⁻¹). GAPDH was used as a loading control. **d**, Annexin-V/7-
17 AAD flow cytometric staining of SUM149 cells treated with Birinapant (0-1000 nM) ± TRAIL (50 ng
18 mL⁻¹) (n=2). **e**, Viability as determined by trypan blue exclusion assay of SUM149 wtXIAP cells treated
19 with TRAIL (50 ng mL⁻¹) and Birinapant or GT13402 (0-1000 nM). Bars represent mean±SEM viable
20 cells taken as a percentage of total cells (n=2-3; *P<0.05, #P<0.005, all comparisons made to untreated)
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39 **Fig 2** *Pan-IAP antagonism is important for Smac mimetic-induced apoptosis in SUM190 cells.* **a**, Western
40 immunoblot analysis showing TRAIL receptor DR4 expression in SUM190 and SUM149 cells. GAPDH
41 was used as a loading control. **b**, Graph, Viability as determined by trypan blue exclusion assay of
42 SUM149, SUM190 and SUM190 shXIAP after treatment with TRAIL (0-100 ng mL⁻¹) at 24 h. Data
43 represent mean±SEM viable cells taken as a percentage of total cells (n=3-5; #P<0.005, all comparisons
44 made between SUM190 and SUM190 shXIAP at the same concentration of TRAIL). Immunoblot, XIAP
45 expression in SUM190 FG12 vector control and SUM190 shXIAP cells. Actin was used as a loading
46 control. **c**, Top panel, SUM190 cell viability as determined by trypan blue exclusion assay when treated
47 with Birinapant or GT13402 (0-10,000 nM). Lower panel, Viability of SUM190 shXIAP cells as
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4 determined by trypan blue exclusion assay treated with Birinapant or GT13402 (0-10,000 nM). Data
5 represent mean±SEM viable cells taken as a percentage of total cells (n=3-5; * P <0.05, all comparisons
6 made between Birinapant and GT13402 at the same concentration). **d**, Western immunoblot analysis of
7 cIAP1, XIAP, and PARP expression, and JNK phosphorylation status in SUM190 cells treated with
8 Birinapant (0-1000 nM) for 4 h. GAPDH and total JNK were used as loading. **e**, controls Annexin-V/7-
9 AAD flow cytometric staining of SUM190 cells treated with Birinapant (B; 0-1000 nM) or TRAIL (100
10 ng mL⁻¹) (n=2; # P <0.005; all comparisons made to the untreated sample based on total Annexin-V
11 staining). **f**, Western immunoblot analysis of total NF-κB and phosphorylation status in SUM190 cells
12 treated with Birinapant (0-1000 nM) for 4, 8, 12, or 24 h, as indicated. GAPDH was used as a loading
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27 **Fig 3** *Smac mimetics inhibit clonogenic growth potential in combination with TRAIL in SUM149 cells*
28 *and as single agents in SUM190 cells.* **a**, Clonogenic growth assay in SUM149 cells treated with TRAIL
29 (10 ng mL⁻¹) alone or in combination with Birinapant (0-1000 nM). Bars represent mean±SEM colonies
30 formed/cells plated as a percentage of the untreated sample. Representative images are shown on the
31 right. **b**, Clonogenic growth assay in SUM149 cells treated with TRAIL (50 ng mL⁻¹) alone or in
32 combination with Birinapant or GT13402 (0-1000 nM). Bars represent mean±SEM colonies formed/cells
33 plated as a percentage of the untreated sample. Representative images are shown on the right. **c**,
34 Clonogenic growth assay in SUM190 cells treated with Birinapant, GT13402 (0-10,000 nM) or TRAIL
35 (0-100 mL⁻¹). Bars represent mean±SEM colonies formed/cells plated as a percentage of the untreated
36 sample (* P <0.05, # P <0.005; all comparisons made to the untreated sample). Representative images are
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53 **Fig 4** *Birinapant inhibits anchorage independent growth potential in combination with TRAIL in SUM149*
54 *cells and as a single agent in SUM190 cells.* **a**, Anchorage-independent growth assay in SUM149 cells
55 treated with TRAIL (50 ng mL⁻¹), Birinapant (1000 nM), or the combination. Bars represent mean±SEM
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4 colonies formed in soft agar as a percentage of the untreated sample ($#P<0.005$; comparisons made to
5 TRAIL alone). Representative images are shown on the right. **b**, Anchorage-independent growth assay of
6 SUM190 cells treated with Birinapant (0-10,000 nM). Bars represent mean \pm SEM colonies formed in soft
7 agar as a percentage of the untreated sample ($#P<0.005$; all comparisons made to the untreated sample).
8 Representative images are shown on the right
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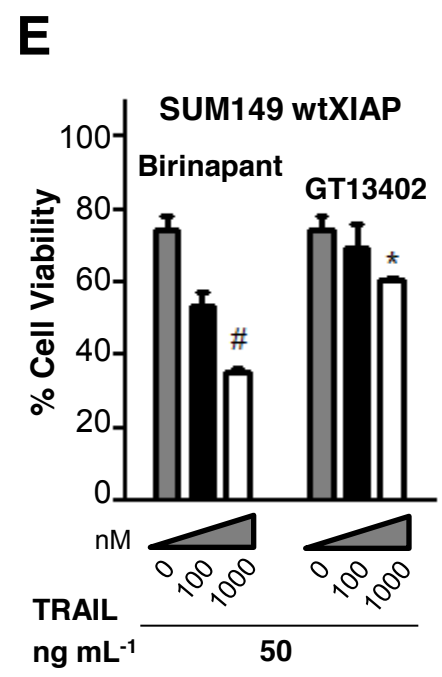
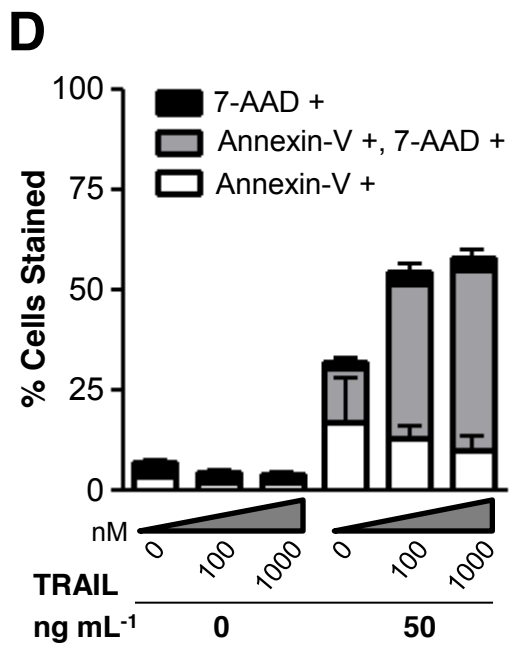
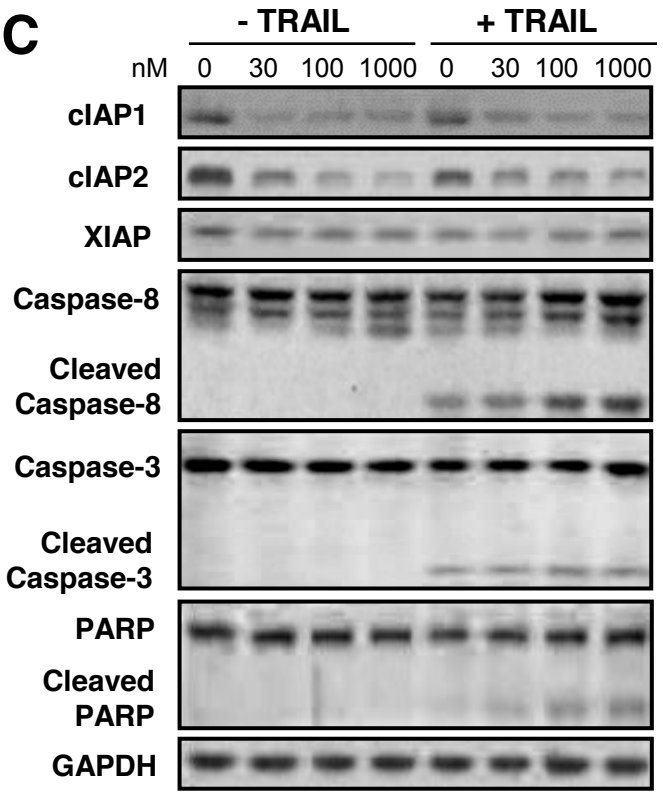
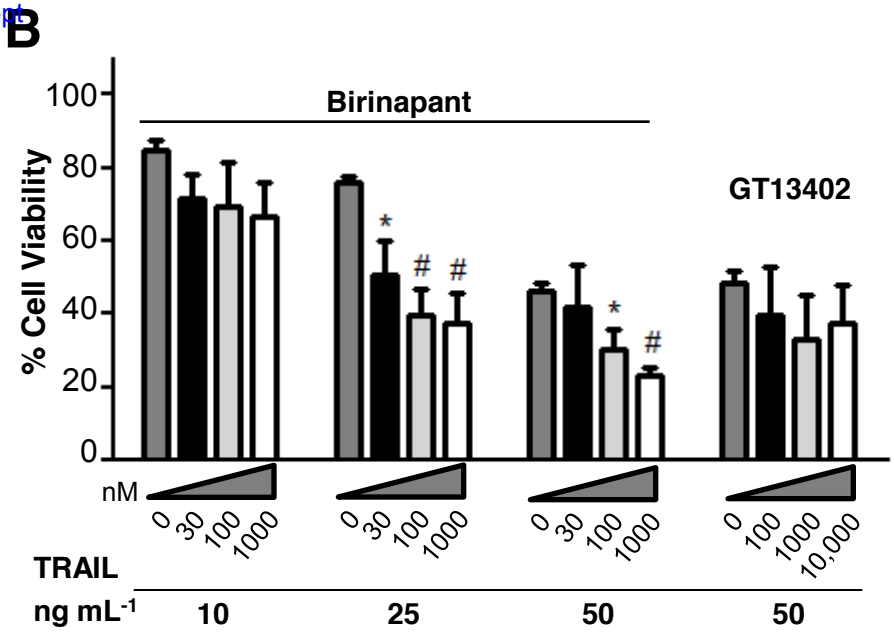
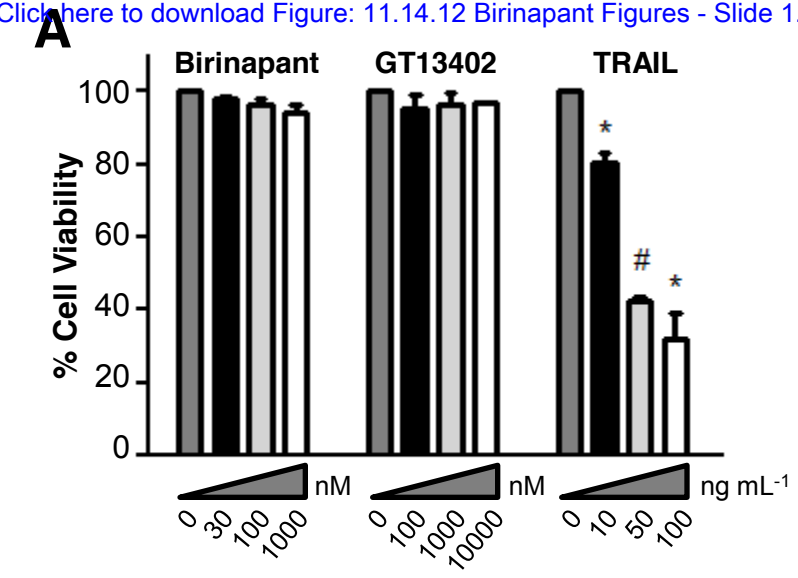
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16 **Fig 5** *SUM190 cells produce low levels of autocrine TNF- α after treatment with Birinapant, but*
17 *exogenous TNF- α does not sensitize SUM190 cells to Birinapant. a*, TNF- α production as determined by
18 ELISA in conditioned media from SUM149 and SUM190 cells treated with Birinapant (0-1000 nM). Bars
19 represent mean \pm SEM (n=3). **b**, Viability as determined by trypan blue exclusion assay of SUM190 cells
20 treated with Birinapant (0-1000 nM) alone or in combination with TNF- α (50 ng mL⁻¹). Data represent
21 mean \pm SEM viable cells taken as a percentage of total cells (n=2-4). **c**, Clonogenic growth assay of
22 SUM190 cells treated with Birinapant (B; 0-1000 nM) alone or in combination with TNF- α (50 ng mL⁻¹).
23 Bars represent mean \pm SEM colonies formed/cells plated as a percentage of the untreated sample
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35 **Fig 6** *Inhibition of TNF- α signaling through TNFR1 knockdown does not inhibit Birinapant-mediated cell*
36 *death in SUM190 cells. a*, Viability as measured by trypan blue exclusion assay of SUM190 cells with
37 TNFR1 knockdown via siRNA or control siRNA treated with Birinapant (0-1000 nM). Data represent
38 mean \pm SEM viable cells taken as a percentage of total cells (n=1-4). **b**, Western immunoblot analysis of
39 TNFR1 in control SUM190 cells (C) and those transfected with TNFR1-targeting siRNA or scrambled
40 siRNA (scr). GAPDH was used as a loading control
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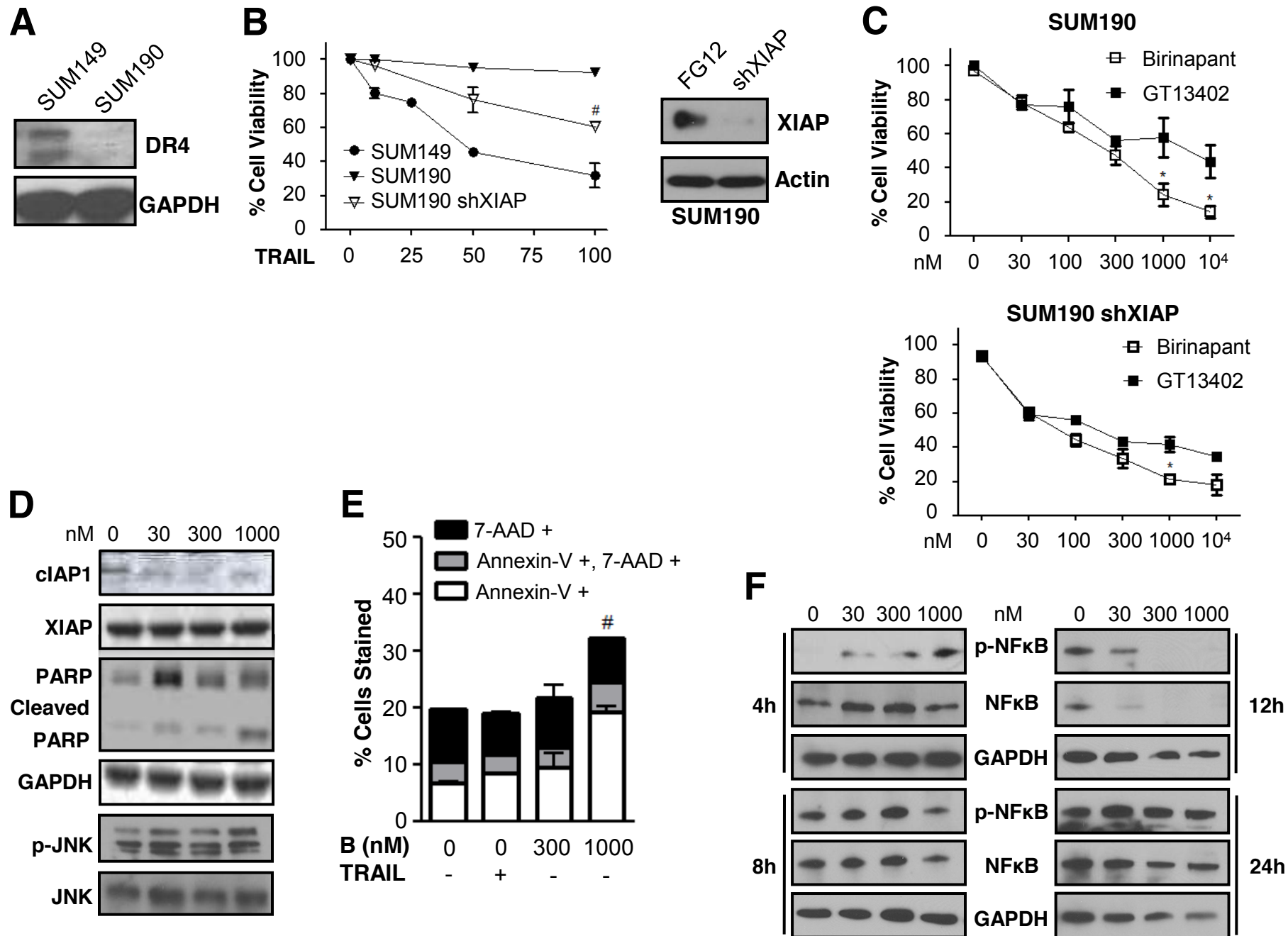
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50 **Fig 7** *Birinapant acts in a TNF- α -independent, caspase-dependent manner to kill SUM190 cells as a*
51 *single agent and sensitize SUM149 cells to TRAIL. a*, Viability as determined by trypan blue exclusion
52 assay of SUM149 cells treated with TRAIL (50 ng mL⁻¹) in combination with Birinapant (0-1000 nM)
53 with or without a TNF- α neutralizing antibody (10 μ g mL⁻¹) or pan-caspase inhibitor Q-VD-OPh (20
54 μ M). Data represent mean \pm SEM viable cells taken as a percentage of total cells (n=2-4, $**P<0.01$; all
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4 comparisons made between Birinapant+TRAIL and Birinapant+TRAIL+TNF- α Ab or Q-VD-Oph at the
5 same concentration of Birinapant). **b**, Clonogenic growth assay of SUM149 cells treated with TRAIL (50
6 ng mL⁻¹) in combination with Birinapant (1000 nM) with or without TNF- α Ab (10 μ g mL⁻¹) or pan-
7 caspase inhibitor Q-VD-Oph (20 μ M). Bars represent mean \pm SEM colonies formed/cells plated as a
8 percentage of the untreated sample. ($\#P<0.005$; all comparisons made between Birinapant+TRAIL and
9 Birinapant+TRAIL+TNF- α Ab or Q-VD-Oph at the same concentration of Birinapant). **c**, Viability as
10 determined by trypan blue exclusion assay of SUM190 cells treated with Birinapant (0-1000 nM) in the
11 presence of a TNF- α neutralizing antibody (10 μ g mL⁻¹) or the pan-caspase inhibitor Q-VD-Oph (20 μ M).
12 Data represent mean \pm SEM viable cells taken as a percentage of total cells (n=2-4, * $P<0.05$, ** $P<0.01$; all
13 comparisons made between untreated and Q-VD-Oph-treated at the same concentration of Birinapant). **d**,
14 Clonogenic growth assay of SUM190 cells treated with Birinapant (1000 nM) in the presence of a TNF- α
15 neutralizing antibody (10 μ g mL⁻¹) or the pan-caspase inhibitor Q-VD-Oph (20 μ M). Bars represent
16 mean \pm SEM colonies formed/cells plated as a percentage of the untreated sample. (* $P<0.05$; all
17 comparisons made between TNF- α neutralizing antibody or Q-VD-Oph-treated at the same concentration
18 of Birinapant)
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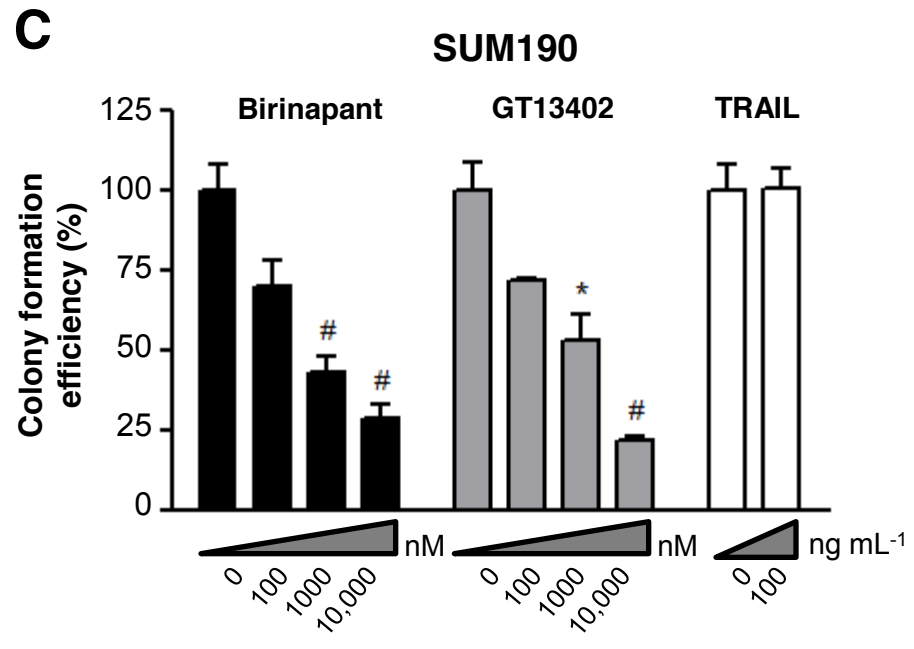
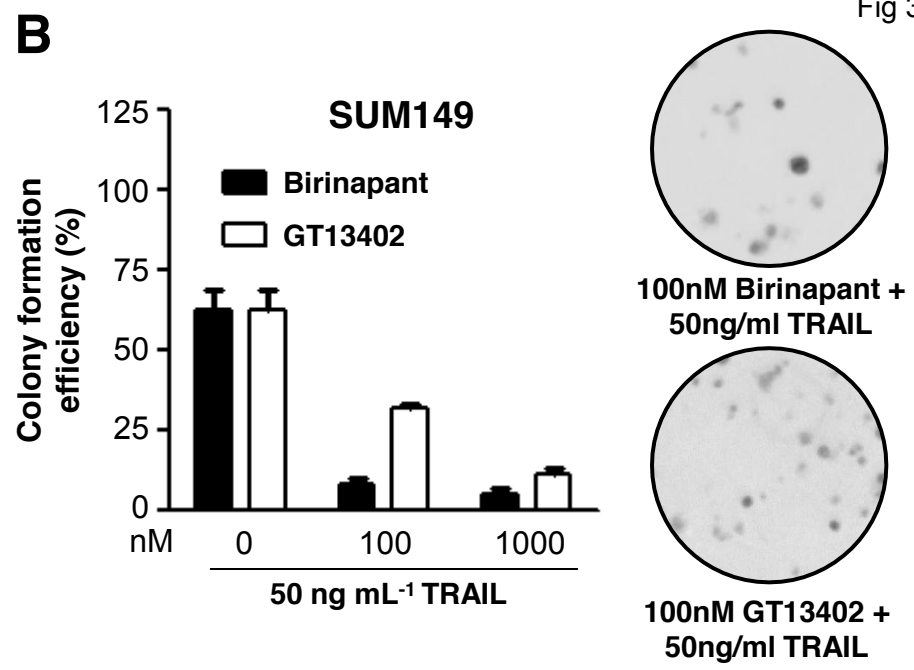
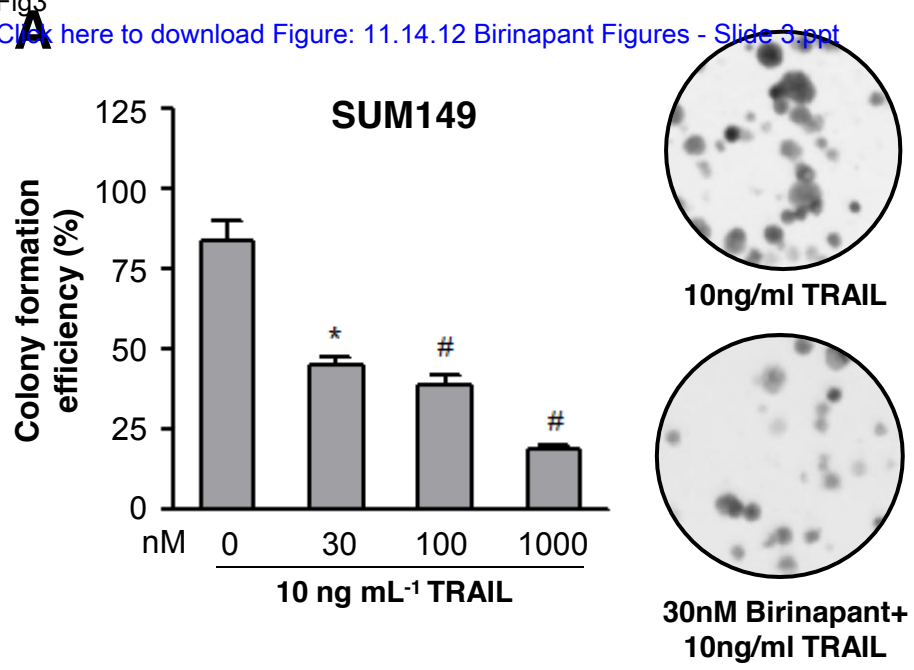
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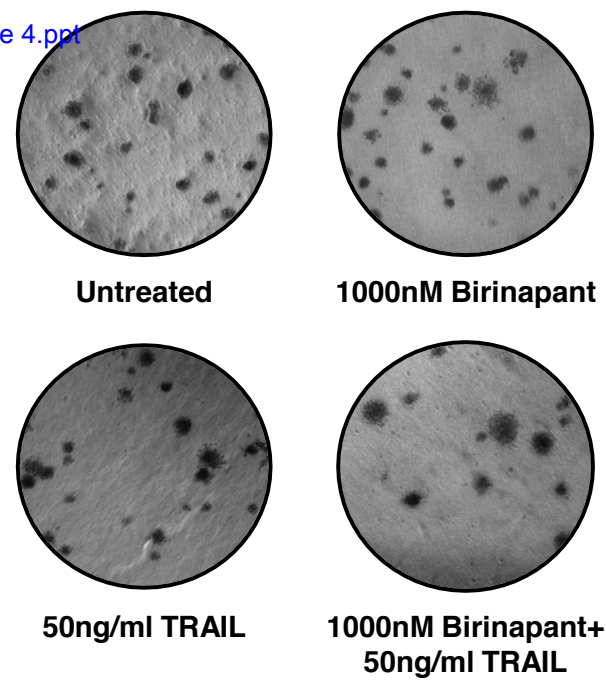
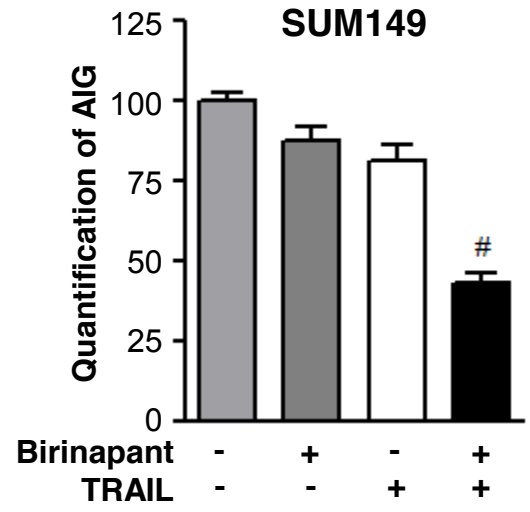


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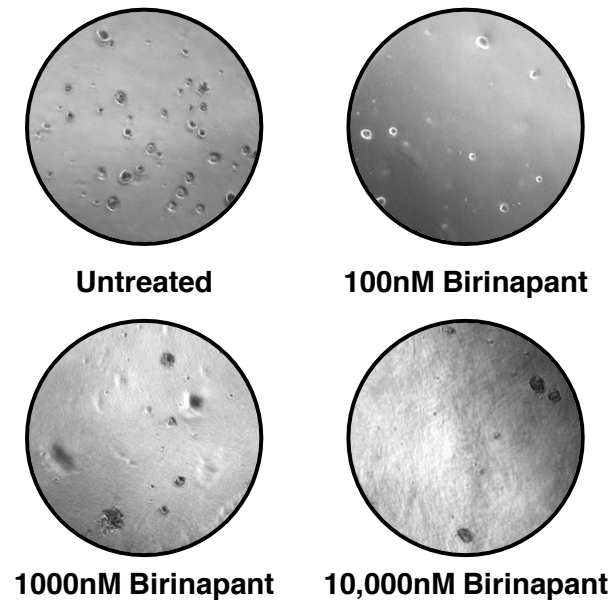
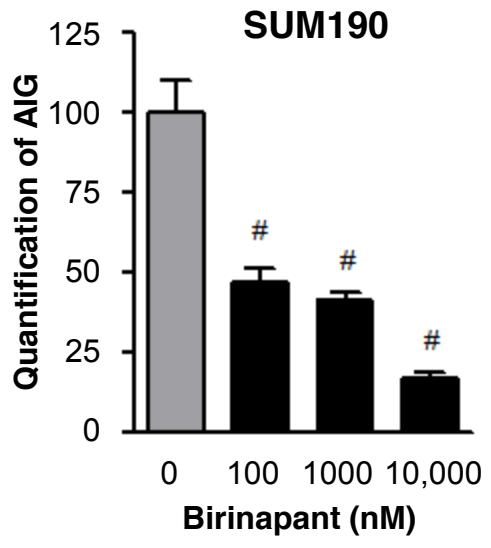


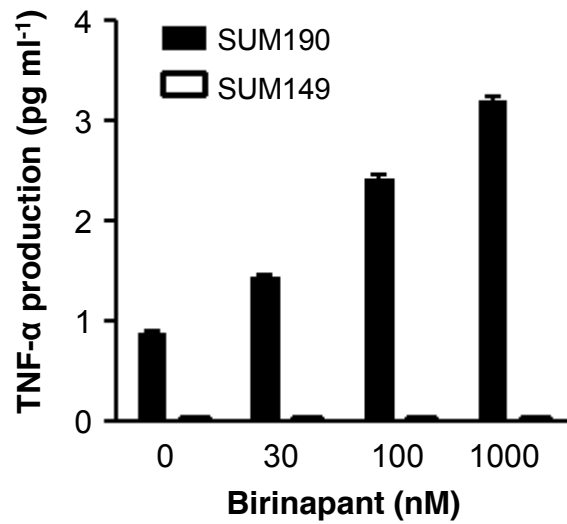
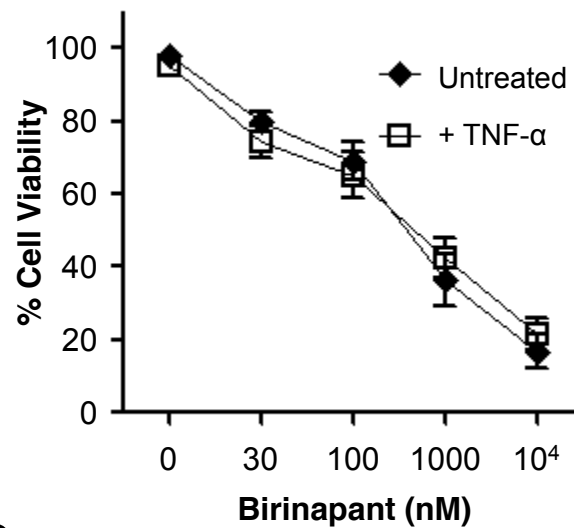
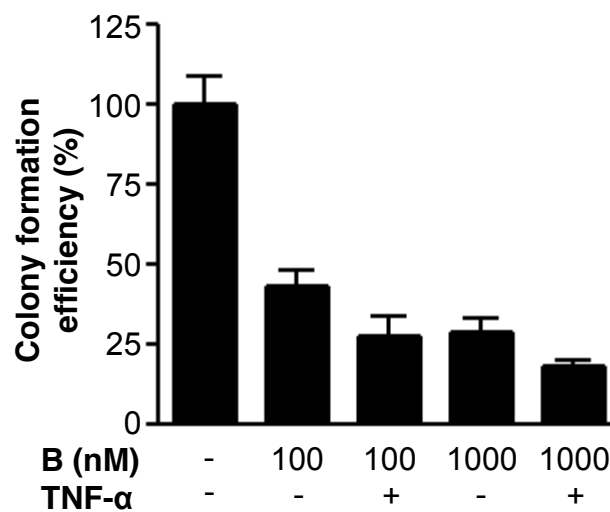
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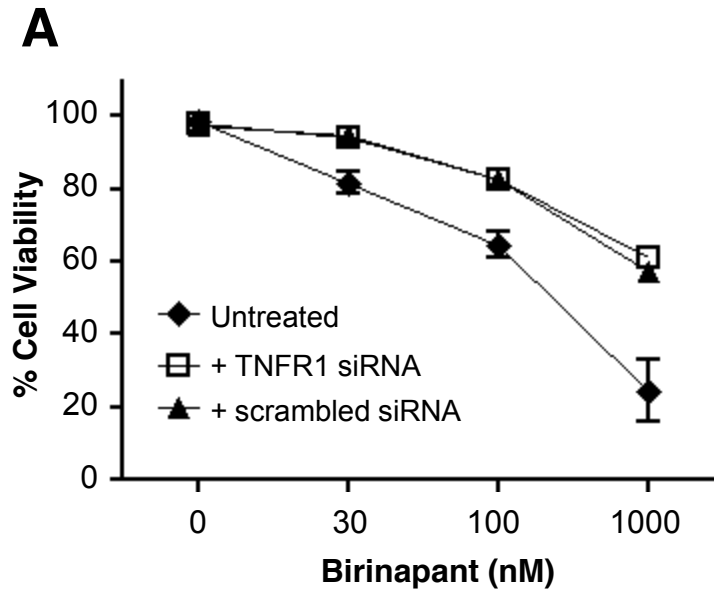
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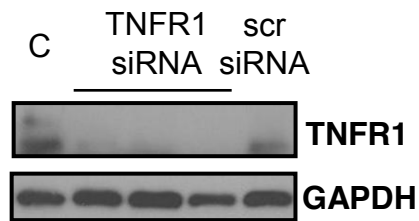
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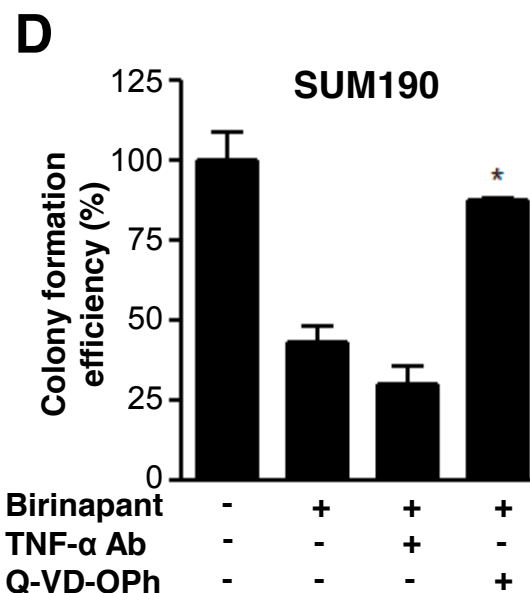
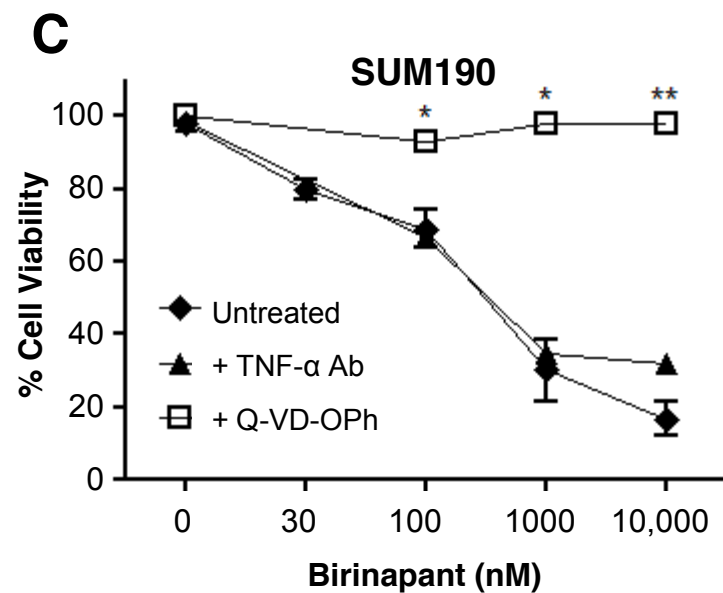
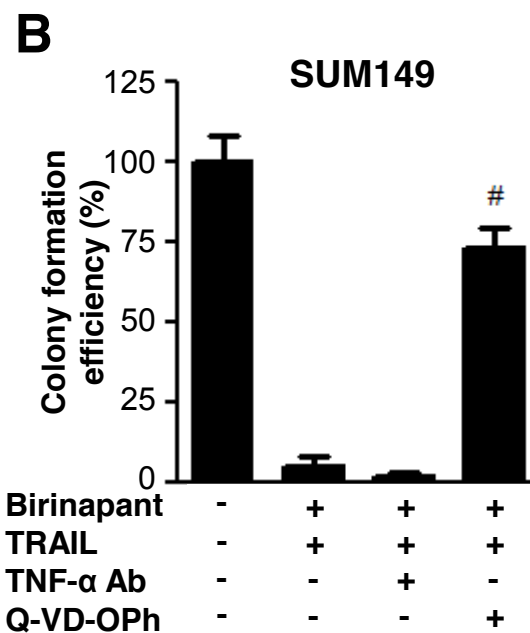
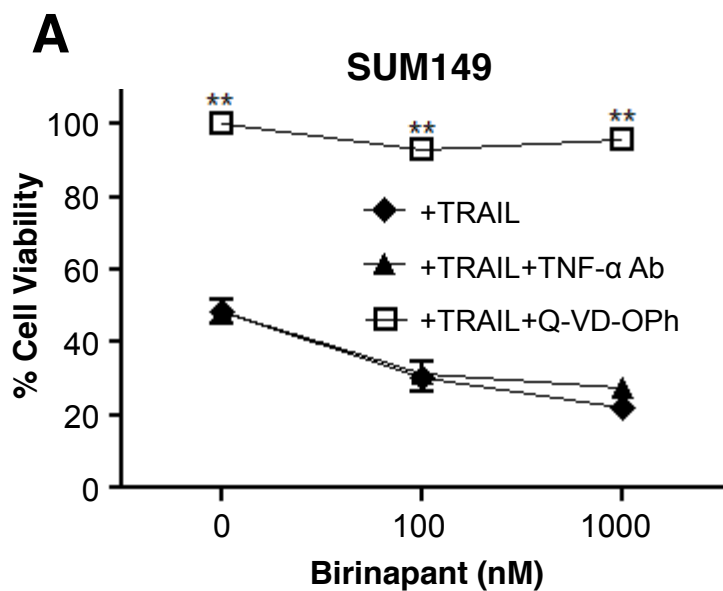
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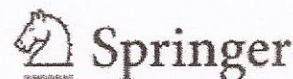


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I have had full access to all the data in the study (if applicable) and thereby accept full responsibility for the integrity of the data and the accuracy of the data analysis.

Page 1 of 2 (Signatures and dates are required on page 2)
BREAST CANCER RESEARCH AND TREATMENT
Authorship/Disclosure Form



MANUSCRIPT ID NUMBER

Completed forms must be scanned and included as a PDF file as part of the online submission process.

Author 1 (printed name): Jennifer L. Allensworth

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- Consultant/advisory role: Entity: _____
- Stock ownership Entity: _____
- Funding Entity: _____

Signature: Jennifer L. Allensworth Date: 07.11.12

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- Stock ownership Entity: _____
- Funding Entity: _____

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