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## Effect of niclosamide on basal-like breast cancers

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### Abstract

Basal-like breast cancers (BLBCs) are poorly differentiated and display aggressive clinical behavior. These tumors become resistant to cytotoxic agents and tumor relapse has been attributed to the presence of cancer stem cells (CSCs). One of the pathways involved in CSC regulation is the Wnt/ $\beta$ -catenin signaling pathway. LRP6, a Wnt ligand receptor, is one of the critical elements of this pathway and could potentially be an excellent therapeutic target. Niclosamide has been shown to inhibit the Wnt/ $\beta$ -catenin signaling pathway by causing degradation of LRP6. TRA-8, a monoclonal antibody specific to TRAIL death receptor 5, is cytotoxic to BLBC cell lines and their CSC enriched populations. The goal of this study was to examine whether niclosamide is cytotoxic to BLBCs, specifically the CSC population, and if in combination with TRA-8 could produce increased cytotoxicity. Aldehyde dehydrogenase (ALDH) is a known marker of CSCs. By testing BLBC cells for ALDH expression by flow cytometry, we were able to isolate a non-adherent population of cells that have high ALDH expression. Niclosamide showed cytotoxicity against these non-adherent ALDH expressing cells in addition to adherent cells from four BLBC cell lines: 2LMP, SUM159, HCC1187 and HCC1143. Niclosamide produced reduced levels of LRP6 and  $\beta$ -catenin, which is a downstream Wnt/ $\beta$ -catenin signaling protein. The combination of TRA-8 and niclosamide produced additive cytotoxicity and a reduction in Wnt/ $\beta$ -catenin activity. Niclosamide in combination with TRA-8 suppressed growth of 2LMP orthotopic tumor xenografts. These results suggest that niclosamide or congeners of this agent may be useful for the treatment of BLBC.

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## Keywords

TRA-8; Tigatuzumab; Death Receptor 5; Basal-like Breast Cancer; Cancer Stem Cells; Tumor Initiating Cells; LRP6; Wnt/ $\beta$ -catenin; Niclosamide (Niclocide)

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## INTRODUCTION

Breast cancer is the second leading cause of cancer-related deaths in North American women. Basal-like breast cancer (BLBC), one of four subtypes of breast cancer, accounts for 13% of all breast carcinomas. While not as common as the other subtypes, BLBC is the most deadly and aggressive type (1, 2). This aggressiveness is due to the fact that it is also the most chemoresistant of the breast cancer subtypes (3). Biologically, it is characterized by a unique mRNA profile with CK5/6 expression and inactivation of *BRCA1*. Additionally, 70–90% of BLBC tumors are characterized as triple negative breast cancer (TNBC) because they lack progesterone and estrogen receptor expression and HER-2 amplification (1, 4, 5). The poor prognosis is linked to its enrichment for tumor initiating cells known as cancer stem cells (CSCs) (6–8). CSCs engage in self-renewal, induce tumors at low-cell density, and produce tumors with differentiated and heterogeneous cell profiles. Moreover, they exhibit gene expression profiles that diverge from their more differentiated cancer cell counterparts. Breast CSCs form tumorspheres *in vitro*, they are more tumorigenic in mice, and more resistant to standard chemotherapy and radiation than differentiated cells (9, 10). In BLBC, CSCs are identified by their extracellular expression of CD44+/CD24- and elevated enzymatic activity of aldehyde dehydrogenase (ALDH) (11, 12). These CSCs are also identified based on the aberrant regulation of their self-renewing pathways, including Wnt, Hedgehog, and Notch signaling (11, 13).

One promising approach to prevent BLBC recurrence and metastasis is to target pathways that regulate CSCs such as the Wnt/ $\beta$ -catenin pathway (3, 14). The cell surface receptor LRP6, essential for Wnt/ $\beta$ -catenin signaling, is a potential target as its expression is up-regulated in 20–36% of human breast cancers and most significantly in the BLBC subtype. Suppression of LRP6 has been proven to be sufficient in inhibiting the Wnt/ $\beta$ -catenin signaling pathway in breast cancer; therefore, it is an excellent potential target for the treatment of BLBC (14–17).

Wnt proteins activate the Wnt/ $\beta$ -catenin pathway by binding to its surface receptors LRP5/6. This binding induces the receptors to interact with the transmembrane receptor, Frizzled (Fz), which leads to the subsequent phosphorylation of LRP5/6 (18). This leads to a build-up of  $\beta$ -catenin, an intracellular signal transducer, in the cytoplasm.  $\beta$ -catenin can then translocate to the nucleus, where it interacts with T-cell transcription factor (TCF). This interaction triggers the transcription of the Wnt pathway target genes, which include survivin, Axin2 and cyclin D1. The expression of these genes leads the cell to undergo proliferation, self-renewal and survival. In the absence of a Wnt ligand,  $\beta$ -catenin is tagged for degradation by the “destruction complex” comprised of adenomatous polyposis coli, Axin and GSK3 $\beta$ , thereby rendering the  $\beta$ -catenin target genes transcriptionally inactive.

The Wnt/ $\beta$ -catenin pathway can be inhibited at the extracellular level by secreted inhibitors such as DKKs or SFRPs (19, 20). Chemically this inhibition can be achieved by salinomycin or niclosamide, which both are able to inhibit the binding of a Wnt ligand to LRP5/6 receptors (21, 22). Inhibitors of Wnt/ $\beta$ -catenin signaling, such as niclosamide, are reported to stimulate Fz internalization and promote LRP6 degradation, thus preventing proliferation and causing apoptosis (22–24). Niclosamide (trade name Niclocide) is a teniacide in the antihelminth family that has been FDA approved for the treatment of tapeworms. This safe,

inexpensive drug has been used in humans for nearly 50 years (25). Niclosamide has also been shown to be cytotoxic against prostate cancer, colorectal cancer, myelogenous leukemia, and ovarian cancer; in ovarian cancer it has been specifically shown to suppresses CSCs (24, 26–28).

Wnt/ $\beta$ -catenin signaling is also inhibited by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) specifically by promoting caspase 3 and 8 mediated cleavage of  $\beta$ -catenin (29, 30). TRAIL also preferentially induces apoptosis in BLBC (31). TRA-8 is an agonistic monoclonal antibody (mAb) to TRAIL death receptor 5 (DR5) (32, 33). We have previously shown that TRA-8 can kill both parental and CSCs from BLBC (34, 35). Furthermore, niclosamide has been shown to reduce the expression of the transcription factor Signal Transducer and Activator of Transcription 3 (STAT3) (36). This is important for our studies since STAT3 plays a key role in many cellular processes such as cell growth and apoptosis, and in breast cancer, STAT3 has been shown to be constitutively activated (37). More recently, it has been described that in breast CSCs, the STAT3 pathway plays a critical role in the conversion of non-CSCs into CSCs through regulation of OCT-4 gene expression (38). Based on this information, we hypothesized that the suppression of canonical Wnt/ $\beta$ -catenin and STAT3 activity by niclosamide would have cytotoxic potential alone and would sensitize BLBC stem cells to treatment with TRA-8. In this study, we explored whether BLBC cell lines, non-adherent ALDH enriched (NAAE) cells, and cells isolated from BLBC patient pleural effusion samples were sensitive to niclosamide alone or in combination with TRA-8.

## MATERIALS AND METHODS

### Drugs and Antibodies

Niclosamide was purchased from Sigma (St. Louis, MO). Niclosamide for *in vitro* use was dissolved in DMSO at a 4.8 mM concentration and stored at 4°C until further use. For animal studies, niclosamide was dissolved in DMSO until a homogeneous suspension was observed at which time Cremophor was added to make a final solution of 25% DMSO and 75% Cremophor. The liquid was slowly inverted to obtain a clear orange solution, which was stored at 4°C. Purified TRA-8 (IgG1) mAb was prepared at the University of Alabama at Birmingham, as described previously, and was provided by Dr. Tong Zhou (32). IgG1 and isotype-specific IgG1 control antibody were obtained from Southern Biotechnology Associates (Birmingham, AL). ALDEFLUOR kit including DEAB was obtained from StemCell Technologies (Durham, NC). Monoclonal anti-phosphorylated-LRP6, Axin2, cyclin D1, p(Tyr705)-STAT3 and STAT3 were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Monoclonal anti- $\beta$ -catenin was purchased from BD Biosciences (San Jose, CA). Survivin antibody for Western blots was purchased from Santa Cruz (Santa Cruz, CA).

### Cells, Cell Culture and Patient Pleural Effusion Samples

The 2LMP subclone of the human breast cancer cell line MDA-MB-231 was obtained from Dr. Marc Lippman (University of Miami, Miami, FL) and maintained in Improved-MEM supplemented with 10% FBS (Hyclone, Logan, UT). BLBC cell lines: HCC1187 and HCC1143 were obtained from American Type Culture Collection (Manassas, VA) and cultured according to supplier's directions. SUM159 was obtained from Asterand (Detroit, MI) and grown according to supplier's recommendation. MCF10A immortalized, non-transformed epithelial cells were obtained from American Type Culture Collection. Cell lines were obtained three years ago, frozen in aliquots which were passaged no more than 15 times and then a new aliquot was used. They were not authenticated. All cell lines were

maintained in antibiotic-free medium at 37°C in a 5% CO<sub>2</sub> atmosphere and routinely screened for mycoplasma contamination.

With Institutional Review Board (IRB) approval, pleural effusion fluid was collected from advanced stage TNBC cancer patients. Cells were washed in phosphate buffered saline (PBS) (HyClone, Logan, UT) and isolated via centrifugation at 400 rpm × 5 minutes. Cells were then plated in ultra-low attachment plates (Corning, Corning, NY) and incubated in mammary epithelial cell growth medium (MEGM) (Lonza, Walkersville, MD).

### Generation and Characterization of Tumorspheres and NAAE Cells

BLBC cells were seeded in 96-well ultra-low attachment plates (Corning) at 40,000 cells/mL in mammary epithelial basal medium (MEBM) supplemented with bullet kit (bovine pituitary extract, insulin, human recombinant epidermal growth factor and hydrocortisone); complete media is called MEGM (Lonza). These non-adherent cells then formed tumorspheres. Cells were tested for ALDH activity at 6, 12, 24, 48, and 72 hours after plating by flow cytometry as previously described (34). In addition, cells were sorted for the ALDH<sup>+</sup> population and retention of ALDH was analyzed at the same time-points. Both sorted and non-sorted cells were found to be maximally enriched for ALDH at 12 hours, but then decreased at later time-points. The NAAE cells obtained at the 12 hour time-point (20,000 cells) were injected into the mammary fat pad (MFP) of NOD/SCID mice. The non-adherent cells at 72 hours after plating (20,000 cells) were also injected into the MFP of mice. To evaluate the difference between the tumorigenicity of the NAAE cells (12 hours after plating) and the non-adherent cells (72 hours after plating), the tumors were measured twice weekly until mice were euthanized in accordance to IACUC regulations.

### Generation of LRP6 Knockdown Cell Line

LRP6 protein expression was knocked down (KD) in the adherent 2LMP cell line using Mission Lentiviral transduction particles SHCLNV, MOI 1 of the TRCN0000033405 (Lot 01091309MN, 1.5×10<sup>7</sup> TU/mL). We used Mission transduction particles for non-mammalian shRNA as a control, SHC002V, MOI 3 (Lot 04301208MN, 2.6×10<sup>7</sup> TU/mL) (Sigma-Aldrich, St. Louis, MO). Cells were selected using ready-made solution puromycin dihydrochloride (Sigma-Aldrich). LRP6 KD was confirmed using Western blot analysis of LRP6.

### Western Blotting

To evaluate the difference in Wnt/β-catenin pathway signaling in NAAE cells and adherent cells, adherent and NAAE 2LMP cells were seeded (1 × 10<sup>6</sup> cells/well) in 6-well plates. Cells were lysed in 0.5 ml of lysis buffer (PBS containing 1% Triton X-100 and 1 mM PMSF) at 4°C for 15 min. Equal quantities of protein were subjected to SDS-PAGE under reducing conditions. Following transfer to immobilon-P membrane, successive incubations with anti-LRP6, anti-p-LRP6, anti-free β-catenin, anti-total β-catenin or anti-actin, and horseradish peroxidase-conjugated secondary antibody were carried out for 60–120 minutes at room temperature. The immunoreactive proteins were then detected using the ECL system (PerkinElmer, Waltham, MA). Films showing immunoreactive bands were scanned by Kodak Digital Science DC120 Zoom Digital Camera (Kodak, Rochester, NY). Cytosolic free β-catenin-analysis was done with GST-E-cadherin binding assay (22). Uncomplexed cytosolic free β-catenin in 100 mg of total cell lysate was subjected to SDS-PAGE and detected using the mAb to β-catenin.

For treatment studies, 2LMP adherent and NAAE cells, and patient samples were seeded (1×10<sup>6</sup> cells/well) in 6-well plates and treated for 24 hours with niclosamide (0, 0.5, 0.25, 0.125 μM). Additional proteins detected were Axin 2, survivin, cyclin D1, p(Tyr705)-

STAT3, and STAT3. 2LMP and HCC1187 adherent and NAAE cells were seeded in the same manner and treated with TRA-8 alone (25 and 125 ng/ml) for 2 hours. Anti-total  $\beta$ -catenin and anti-actin antibodies were used.

### TOPflash Luciferase Reporter Assay

NAAE cells from all four BLBC cell lines were plated at 40,000 cells/well. The next day, the cells were transfected with 0.05 mg of TCF/LEF activity reporter (TOPflash) (plasmid from Dr. Randall Moon's laboratory, Upstate Biotechnology, Lake Placid, NY). Cells were transfected using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) in Opti-MEM (Gibco/Invitrogen) as per manufacturer's instructions. Six hours after transfection, cells were treated with 0.25  $\mu$ M niclosamide for 24 hours. Adherent BLBC cells were plated and transfected in the same manner. In one experiment, adherent cells were treated with 0.25  $\mu$ M niclosamide for 48 hours and 0.25 ng/mL TRA-8 for 24 hours. In another experiment, adherent SUM159, HCC1143, and HCC1187 cells were treated with 1  $\mu$ M niclosamide and 2LMP cells with 0.25  $\mu$ M niclosamide for 24 hours. Each cell line was also concurrently treated with Wnt3A (5 ng/ml) (R&D Systems, Minneapolis, MN) alone and in combination with niclosamide.

Patient samples were plated in the same manner and transfection was attempted with the plasmid, but obtaining a consistently high transfection efficiency for the analysis proved challenging. Therefore, the method was revised to use lentivirus pCignal Lenti TCF/LEF Reporter at MOI of 25 (Qiagen, Germantown, MD) to transfect the cells. Twenty-four hours after transfection with the lentivirus, cells were treated with 4  $\mu$ M niclosamide for 24 hours. Total protein was harvested 24 hours post-treatment and luciferase activity was measured using a Turner 20/20 luminometer (Promega, Madison, WI). The luciferase reading was normalized to the total protein concentration as reported previously (39). The luciferase activity was normalized to untreated control and represented as a mean  $\pm$  SE for a minimum of three replicates.

### Tumorsphere Inhibition Assay

NAAE cells for all four BLBC lines were plated at 2,000 cells/well in 50  $\mu$ L media, then treated with niclosamide (0.25–1.0  $\mu$ M) for 24 hours, followed by treatment with TRA-8 (1 ng/mL 2LMP, 0.5 ng/mL SUM159, 25 ng/mL HCC1143, and 5 ng/mL HCC1187) for an additional 24 hours. Two independent investigators used a reticle eyepiece to visually count tumorspheres. Mean tumorsphere inhibition was calculated relative to no treatment. Samples were run in quadruplicate in three independent experiments and error bars represent  $\pm$  standard error (SE).

### *In vitro* Cytotoxicity Studies

2LMP, SUM159, HCC1143, HCC1187, NAAE and attached cells, MCF10A, LRP6 KD, or shRNA attached cells were seeded at 2,000 cells/50  $\mu$ L of media. Adherent cells were plated in optically clear tissue-culture-treated black plates (Costar, Corning, NY). NAAE cells and patient samples were plated in low-attachment plates in MEGM media. To assess the viability of cells exposed to niclosamide or TRA-8, all cells were treated with niclosamide (0.125–8  $\mu$ M) or TRA-8 (0.1–500 ng/mL) for 24 or 48 hours. In combination treatment studies, after 24 hours exposure to niclosamide, TRA-8 (0.25–500 ng/mL) was added for an additional 24 hours before the cells were lysed and analyzed for viability using the ATPlite luminescence-based assay (PerkinElmer) as previously described (40). Alternatively, cells were treated concurrently with niclosamide and TRA-8 for 48 hours. TRA-8 and niclosamide were diluted in culture medium immediately before use, and the final concentration of DMSO was always 0.001%. All samples were assayed in triplicate or quadruplicate and are reported as the mean  $\pm$  SE.

## **In Vivo Therapy and Toxicity Studies**

Female athymic nude mice at 4 weeks of age were purchased from Harlan (Indianapolis, IN). *In vivo* generation of tumors was accomplished by resuspending  $2 \times 10^6$  2LMP cells in 100  $\mu$ L (1:1 Matrigel and PBS) and injecting cells into the MFP of athymic nude mice (n=5). Mice were randomly assigned into four groups and treated when the tumors reached  $\sim 16 \text{ mm}^2$  (7 days after implantation). Mice were intraperitoneally injected twice weekly with TRA-8 (200  $\mu$ g), niclosamide (30 mg/kg; 50% cremophore EL:50% DMSO) 5 days a week, the combination, or with vehicle control (50% cremophore EL:50% DMSO) 5 days a week for 3 weeks. Tumor size (surface area) was calculated by  $a \times b$ , where  $a$  is the largest diameter and  $b$  is the diameter perpendicular to  $a$ . Tumors were measured with a Vernier caliper 2 times a week until mice were euthanized in accordance with the IACUC regulations.

To assess tumor and normal tissue toxicity a separate animal study was conducted with three mice per group and treated with vehicle control 5 days a week, niclosamide (20 mg/kg) 5 days a week, TRA-8 (200  $\mu$ g) twice weekly and the combination for a total of two weeks. All animals were monitored daily and weighed twice a week. After two weeks of treatment, animals were euthanized and tumor tissue, large and small intestines, kidney, spleen, and liver were dissected and fixed in 10% neutral buffered formalin at room temperature and then processed to paraffin blocks. The tissues from each of the four groups were coded and hematoxylin and eosin stains were performed on 4 $\mu$  tissue sections. The board certified diagnostic pathologist who evaluated the tissues for toxicity was blinded as to the treatment group. In addition, portions of the tumors were evaluated for changes in protein expression by Western blot analysis.

## **Immunohistochemistry**

Patient pleural effusion samples were stained by immunohistochemistry for LRP6, Moc31, and calretinin (Cell Signaling Technology, Inc.). Mouse tumor tissue was also analyzed for p-STAT3 and  $\beta$ -catenin expression. Cells were harvested into histogel and embedded into a paraffin block. For each sample, the staining intensity (0, 1+, 2+, 3+) and the percentage of cells staining positive (0–100%) was determined by a blinded board-certified pathologist. An H-score was calculated as the product of the intensity and the percentage of cells with positive staining.

## **Statistical Analysis**

T-tests were used to compare means when appropriate. General linear models were used to make comparisons over time. Enhanced combination effect was further confirmed by a concentration–effect curve using the nonlinear regression method and isobologram methods (data not shown) (41). Experimental animal treatment groups were composed of 5 animals each to provide evidence of substantial tumor sensitivity to TRA-8 therapy with or without niclosamide. For xenograft models, tumor-doubling time (TDT) was estimated for each animal using empirical distribution, and median TDT between treatment groups was compared using Kruskal-Wallis nonparametric statistical test.

# **RESULTS**

## **Analysis of cells**

In this study, we investigated four BLBC cell lines: 2LMP, SUM159, HCC1143, and HCC1187. All cell lines were analyzed as adherent cells in normal tissue-culture plates with media that contained serum. In addition, cells were plated in ultra-low attachment plates with MEGM media and were noted to form tumorspheres which are composed of non-

adherent cells. Non-adherent cells were further analyzed for the stem cell marker, ALDH, at various time-points and compared to cells that were sorted for ALDH. Both types of cells were noted to have elevated levels of ALDH 12 hours after being plated, which then decreased with time. The non-sorted and non-adherent cells at the 12 hour time-point were termed NAAE (non adherent aldehyde enriched cells).

### Characterization of NAAE Cells

Cells were tested for ALDH activity at 6, 12, 24, 48, and 72 hours for the 2LMP and SUM159 cell lines and at 6, 12, and 24 hours for the HCC1143 cells. These cells were compared to cells that were sorted for the ALDH<sup>+</sup> population and subsequently analyzed for ALDH expression at the same time-points. Both sorted and non-sorted cells were found to be enriched for ALDH at the 12 hour time-point, but subsequently both populations returned to the pre-sorted expression level; therefore the NAAE cells represent a CSC enriched population at the 12 hour time-point (Fig. 1A). To verify enhanced tumorigenicity of the NAAE cells (12 hours after plating), they were injected into mice and compared to cells that were cultured for 72 hours prior to injection, which had reduced ALDH activity. The NAAE cells formed aggressive large tumors indicating their enrichment for CSCs compared to tumors formed by cells from the 72 hour time-point ( $P = 0.01$ ). NAAE xenografts developed earlier and formed larger tumors over time, indicating enrichment for tumor initiating CSCs (Fig. 1B).

2LMP NAAE cells were also analyzed by Western blot for p-LRP6, LRP6, free and total  $\beta$ -catenin (Fig. 1C) and compared to adherent 2LMP cells. The NAAE cells demonstrated higher free  $\beta$ -catenin and lower LRP6 and p-LRP6 expression. Similar levels of total  $\beta$ -catenin were observed in both NAAE and adherent populations.

### Niclosamide Induces Cytotoxicity to BLBC Cell Lines *In Vitro*

We examined the cytotoxicity of niclosamide against both adherent cells and NAAE cells in BLBC cell lines. All four BLBC adherent cell lines were sensitive to niclosamide with an  $IC_{50}$  range of 0.33 - 1.9  $\mu$ M (Supplementary Table 1). NAAE cell populations from BLBC cell lines were more sensitive to niclosamide than their corresponding adherent cell populations with  $IC_{50}$  values of 0.17 - 0.29  $\mu$ M (Table S1). Statistical significance was not met in the 2LMP NAAE compared to the adherent cells (0.29 vs. 0.44) ( $P > 0.05$ ), but the SUM159, HCC1187 and HCC1143 cell lines had a statistically significant difference in the adherent and the NAAE  $IC_{50}$  values. Adherent MCF10A (non-tumorigenic) mammary epithelial cells showed less than 10% niclosamide-mediated cytotoxicity (Supplementary Fig. S1).

### Niclosamide Inhibits Wnt/ $\beta$ -catenin and STAT3 Signaling in BLBC Cell Lines

To characterize the effect of niclosamide on the Wnt/ $\beta$ -catenin pathway, we performed the signaling TOPflash luciferase reporter assay, which measures the level of nuclear  $\beta$ -catenin, on NAAE cells and adherent cells. Wnt3A ligand was added to the four adherent BLBC cell lines to upregulate the Wnt/ $\beta$ -catenin pathway. Nuclear  $\beta$ -catenin was decreased by niclosamide treatment in all four adherent cell lines (0.25  $\mu$ M in the more sensitive cell line, 2LMP, and 1  $\mu$ M in the other three cell lines) after upregulation by Wnt3A ligand ( $P < 0.05$ ). Three of the four cell lines had niclosamide-mediated Wnt/ $\beta$ -catenin inhibition in the absence of the Wnt3A ligand ( $P < 0.05$ ) (Supplementary Fig. S2). NAAE cells from all four BLBC cell lines showed significant inhibition of Wnt/ $\beta$ -catenin signaling following 24 hours of treatment with 0.25  $\mu$ M niclosamide without the addition of the Wnt3A ligand ( $P < 0.01$ ) (Fig. 2A).

Western blot analysis of Wnt/ $\beta$ -catenin signaling proteins: LRP6, pLRP6, free and total  $\beta$ -catenin, 3 target genes: Axin2, survivin, and cyclin D1, two STAT3 pathway proteins: phosphorylated (tyr705) and total STAT3 after 24 hours treatment with niclosamide (0.125  $\mu$ M - 0.5  $\mu$ M) showed a dose response inhibition of the Wnt/ $\beta$ -catenin and STAT3 pathways in both the adherent and NAAE 2LMP cells (Fig. 2B). Cell death was also calculated for these doses to assure that Western results were not a consequence of cell death. The highest dose of 0.5  $\mu$ M did not induce more than 60% kill of the adherent or the NAAE cells when cells were plated according to the Western blotting protocol (Supplementary Fig. S3). The 0.125  $\mu$ M dose caused almost no cell kill, yet did produce decreases in the Wnt/ $\beta$ -catenin target genes, suggesting that the Wnt/ $\beta$ -catenin pathway is downregulated at non-cytotoxic doses of niclosamide.

### Effect of Niclosamide and TRA-8 Treatment *In Vitro*

Our laboratory has previously published on TRA-8 cytotoxicity to BLBC cells (35) and their CD44+/CD24-/ALDH+ cell sorted populations (34), therefore, we hypothesized that the combination of two CSC targeting agents, TRA-8 and niclosamide, would produce increased cytotoxicity of NAAE cells. NAAE cells were pre-treated with niclosamide for 24 hours followed by 24 hours treatment with TRA-8, and the number of tumorspheres was compared to single agents or no treatment. Tumorsphere formation was inhibited in all four cell lines with niclosamide and TRA-8 alone, but the combination of the two agents produced a significant increase in tumorsphere inhibition compared to either agent alone ( $P = 0.03$ ) (Fig. 3A). Cytotoxicity of niclosamide and TRA-8 was also tested against NAAE cells (Fig. 3B). The cytotoxicity results correlated with the tumorsphere inhibition results in all four cell lines.

### Effect of Niclosamide and TRA-8 on Wnt/ $\beta$ -catenin Signaling

The TCF/LEF plasmid reporter TOPflash assay was used to evaluate the combination of niclosamide and TRA-8 on the Wnt/ $\beta$ -catenin signaling pathway in BLBC adherent cells. The TOPflash assay was also attempted for the combination treatment on NAAE cells, but sufficient transfection was never achieved. All four BLBC cell lines were treated for 48 hours with 0.25  $\mu$ M niclosamide. After the first 24 hours, TRA-8 at 0.25 ng/mL was added. Wnt/ $\beta$ -catenin pathway inhibition occurred after treatment with niclosamide or TRA-8 alone as compared to no treatment (Fig. 4A). Combination treatment produced significantly greater inhibition of the Wnt/ $\beta$ -catenin reporter than single agents in three of the cell lines.

TRA-8, unlike niclosamide, was previously not known to influence Wnt/ $\beta$ -catenin signaling. Western blots were used to examine the effect of TRA-8 on  $\beta$ -catenin. Total  $\beta$ -catenin levels decreased (low exposure) with evidence of fragmentation (high exposure) of the protein after a 2 hour TRA-8 treatment of 2LMP adherent and NAAE cells. However, with HCC1187, TRA-8 treatment only produced a decrease in total  $\beta$ -catenin in NAAE cells (Fig. 4B).

Given our observation that niclosamide's inhibition of the Wnt/ $\beta$ -catenin pathway resulted in reduced expression of survivin, we hypothesized that decreasing LRP6 would enhance the cytotoxicity of the apoptotic agent TRA-8. Knock down of LRP6 using Mission Lentiviral transduction particles shRNA on adherent 2LMP cells produced enhanced TRA-8 mediated cytotoxicity (Fig. 4C). LRP6 KD was confirmed by Western blot, which also showed reduced survivin expression compared to a vector control (Supplementary Fig. S4).

### Effect of Niclosamide and TRA-8 on Established 2LMP Tumors

Given the impressive *in vitro* observations of niclosamide and TRA-8 cytotoxicity with BLBC cell lines, we carried out a combination therapy study in an orthotopic BLBC animal

model. Animals received niclosamide (30 mg/kg five days a week), or TRA-8 (200 µg twice weekly), or both treatments for a total of three weeks and were compared to mice that received a vehicle control. The mice that received TRA-8 alone had a significant inhibition of tumor growth ( $P < 0.05$ ), similar to what had been previously reported by our laboratory (35). Animals treated with niclosamide had similar tumor growth inhibition compared to those treated with TRA-8, while animals treated with the combination of agents had significant tumor growth inhibition compared to untreated controls ( $P < 0.01$ ) and to either single agent alone ( $P < 0.05$ ) (Fig. 5).

To further investigate the potential toxicity of niclosamide and TRA-8 in the mice, we performed a two-week *in vivo* treatment of both single agents and the combination. Although this treatment regimen of niclosamide was 20 mg/kg, there was still a significant decrease in tumor growth in both the single agent treatment and further inhibition with the combination treatment. The weight of the treated mice, both with a single agent and the combination did not decrease over time (Supplementary Fig. S5). In addition, the tissue architecture for the kidney, spleen, liver, small and large intestines was analyzed and found to be normal. Furthermore, the tumors in the mice were analyzed by immunohistochemistry which showed treated mice had reduced expression of p-STAT3, cytosolic and nuclear β-catenin, and increased expression of cleaved caspase 3 with niclosamide, TRA-8, and the combination treatment (Supplementary Table S2). This shows that niclosamide, TRA-8, and the combination repress tumor growth by inhibiting the Wnt/β-catenin pathway, the STAT3 pathway, and by causing apoptosis. Western blot analysis of tumor tissue resulted in reduced expression of survivin in niclosamide treated mice and further reduction with combination treatment (results not shown). These results further demonstrate the combination effect of TRA-8 and niclosamide.

### Primary BLBC Patient Samples Response to Niclosamide and TRA-8

The combination of niclosamide and TRA-8 was further investigated on patient BLBC tumor cells collected from pleural effusions from triple-negative treatment resistant patients. All four samples (UAB01, UAB03, UAB04 and UAB05) stained positive for LRP6 by immunohistochemistry (data not shown). All four patient samples showed significant cytotoxicity when treated with niclosamide for 48 hours (1–8 µM) (Fig. 6A). TOPflash assay using lentiviral reporter was performed on UAB03 and UAB05 because these two samples had adequate number of cells available; whereas UAB01 and UAB04 had too few cells. The decrease in percent luciferase activity in the TCF/LEF viral reporter after 24 hours of treatment with 4 µM niclosamide showed Wnt/β-catenin pathway inhibition in these two samples (Fig. 6B). Patient sample UAB03 also had enough cells for Western blot assay to be performed. Niclosamide demonstrated dramatic inhibition of Wnt/β-catenin pathway proteins (LRP6 and β-catenin) and cyclin D1, as well as reduced p-STAT3 and total STAT3 expression in this patient sample when treated for 24 hours with 4 µM niclosamide (Fig. 6C). When niclosamide and TRA-8 treatment was combined in two patient samples, UAB05 and UAB03, tumor cell cytotoxicity was enhanced compared to TRA-8 alone for patient sample UAB05, and a modest combination effect was seen in patient sample UAB03 (Fig. 6D). The higher concentrations of niclosamide that were needed for cytotoxic, Western, and TOPflash effects in the patient samples compared to the cell lines, may reflect the drug resistance history and metastatic nature of the patient samples. Cells collected from two BLBC patient pleural effusion samples (UAB03 and UAB05) were characterized by immunohistochemistry staining, which indicated that over 50% of cells in the sample were positive for Moc31, a marker for metastatic breast cancer, and negative for calretinin, a marker for mesothelial cells, confirming that the cells were metastatic breast cancer epithelial cells rather than pleural mesothelial cells (data not shown).

## DISCUSSION

Most cancer treatment regimens assume that all cancer cells have equal malignant potential and respond similarly to therapy. More recently, therapies are being designed to target both bulk tumor cells and CSCs with the goal to prevent recurrence and metastasis. Therefore, therapies that target both the CSC maintenance pathways and induce apoptosis in the bulk tumor cell population could potentially result in increased chances for survival. We previously described that TRA-8 kills BLBC cell lines both *in vitro* and in xenograft mouse models and preferentially kills their CSC population. In an effort to improve on this strategy, this study demonstrated that niclosamide, a potent inhibitor of Wnt/ $\beta$ -catenin signaling, produced similar cytotoxicity with bulk tumor cells (attached cells), CSC enriched cell populations (NAAE cells), and primary tumor cells from BLBC patients both alone and in combination with TRA-8.

Because CSCs represent a small fraction of the total tumor cell population, it is difficult to run mechanistic studies with these cells especially by techniques such as Western blot and the TOPflash reporter assay that require a large number of cells. NAAE cells were discovered as a tool to analyze the effect of drugs on CSC enriched populations. Our findings imply that sorting may not be necessary to enrich for CSC characteristics if treatments are conducted within a short time period after plating. Our results also indicate that treatments targeted toward CSCs should not be carried out on sorted populations after 48 hours from sorting due to depletion of initial enrichment for CSC marker expression. *In vivo* tumorigenicity studies functionally validated the enhanced tumor take and growth rate of NAAE cells. Future studies will characterize NAAE cells and look at epithelial-mesenchymal transition and mesenchymal-epithelial transition markers that could potentially influence observed dynamic CSC marker expression (42).

The involvement of Wnt/ $\beta$ -catenin signaling in BLBC and CSCs has been well documented (14). High-throughput screening studies identified niclosamide as a potent Wnt/ $\beta$ -catenin pathway inhibitor (43). Multiple publications by various groups have shown that niclosamide is a potent inhibitor of Wnt/ $\beta$ -catenin signaling (22–24). Like many inhibitors of signaling pathways, niclosamide also affects other pathways, such as NF- $\kappa$ B (26), Notch (44), ROS (26), mTORC1 (36), and STAT3 (45). Niclosamide has been shown to be cytotoxic in cell lines from several cancers such as colon (46), ovarian (28), osteosarcoma (23), myeloma (47), and breast cancer (22). Our objective was to investigate niclosamide's cytotoxicity in BLBC cell lines, and patient pleural effusion samples from BLBC patients. NAAE cells were generated from BLBC cell lines to specifically test the sensitivity of CSCs to niclosamide. The Wnt/ $\beta$ -catenin pathway is known to play an important role in CSCs; the sensitivity of NAAE cells to niclosamide treatment further supports the role of the Wnt/ $\beta$ -catenin pathway in cells enriched for stem cell markers. Furthermore, Western blot analysis of NAAE cells showed that they have reduced levels of LRP6, thus their enhanced sensitivity to niclosamide is not due to overexpression of LRP6. NAAE cells did show elevated levels of free  $\beta$ -catenin, which could contribute to their increased sensitivity to niclosamide. Given there was no elevation in LRP6 or p-LRP6 in the NAAE cells, other pathways such as STAT3 could be the mechanism by which nuclear  $\beta$ -catenin is increased. Prior studies have shown that activation of the STAT3 pathway leads to increased levels of  $\beta$ -catenin (48).

The BLBC cells isolated from patient samples that showed cytotoxicity with niclosamide treatment were previously resistant to *in vitro* treatment with chemotherapy and Notch inhibitors (data not shown). The dose of niclosamide that was needed to cause *in vitro* cytotoxicity of these patient samples was within the range of blood concentrations (0.76 –

18.35  $\mu\text{M}$ ) achieved when healthy patients were given 2 g of niclosamide orally once for the treatment of tapeworms (25).

Both TOPflash results and Western blot analysis showed functional inhibition of the Wnt/ $\beta$ -catenin signaling pathway in BLBC cell lines and patient samples treated with niclosamide. Niclosamide also caused a decrease in p-STAT3, which shows that it effects other pathways in addition to the Wnt/ $\beta$ -catenin pathway involved in tumor initiation and progression. The ability of niclosamide to deplete survivin expression could be the mechanism by which there is an increase in apoptosis when BLBC cells are treated with TRA-8. Survivin normally inhibits caspase-9 activation within the apoptosome, a key step in the apoptotic cascade (49). The 2LMP LRP6 Knock Down cells had enhanced sensitivity to TRA-8, and reduced survivin activity, further validating our hypothesis that inhibition of canonical Wnt/ $\beta$ -catenin signaling can sensitize cells to death receptor mediated apoptosis.

The extrinsic apoptotic pathway has been shown to regulate  $\beta$ -catenin activity by TRAIL's ability to decrease full-length  $\beta$ -catenin expression (30, 50). Although TRAIL can induce apoptosis in tumor cells, many cancer cells develop resistance, which could be overcome by combination treatment.  $\beta$ -catenin can be targeted to sensitize apoptosis-resistant cancer cells. TRA-8 and niclosamide led to reduction of  $\beta$ -catenin expression, coinciding with maximal apoptosis. TRA-8's ability to reduce  $\beta$ -catenin was confirmed by TOPflash reporter assay and Western blot analysis of cleaved  $\beta$ -catenin. Future studies will focus on characterizing the mechanism by which TRA-8 can inhibit  $\beta$ -catenin activity and the results could help further elucidate how TRA-8 preferentially induces apoptosis in CSCs.

Overall, this study provides new insights into the treatment of CSCs from BLBC. It further supports existing literature that niclosamide's inhibition of Wnt/ $\beta$ -catenin signaling and reduction in p-STAT3 produces enhanced cytotoxicity against CSCs. Additionally, the results demonstrate that the combination of niclosamide and TRA-8 targets the CSC subpopulation and potentially prevents recurrence and metastasis in patients with BLBC. The combination of these two agents or similar compounds can potentially be translated into clinical trials, helping BLBC patients who have very few treatment options available to them.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Abbreviations

**ALDH** aldehyde dehydrogenase

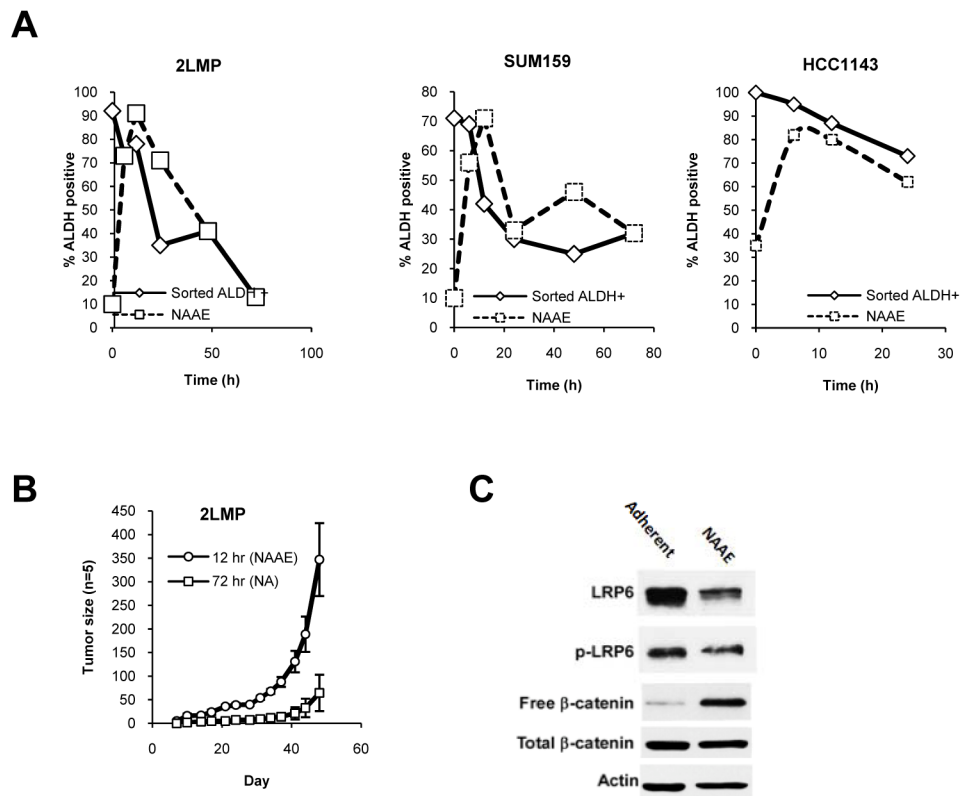
<b>BLBCs</b>	basal-like breast cancers
<b>CK</b>	cytokeratin
<b>CSCs</b>	cancer stem cells
<b>DEAB</b>	diethylaminobenzaldehyde
<b>DKK</b>	dikkopf proteins
<b>DMSO</b>	dimethyl sulfoxide
<b>FACS</b>	fluorescence activated cell sorting
<b>FDA</b>	Food and Drug Administration
<b>Fz</b>	frizzled
<b>IACUC</b>	Institutional Animal Care and Use Committee
<b>IP</b>	intraperitoneal
<b>IRB</b>	Institutional Review Board
<b>KD</b>	knocked down
<b>LEF</b>	lymphoid enhanced factor
<b>LRP</b>	low density lipoprotein receptor-related protein
<b>mAb</b>	monoclonal antibody
<b>MEBM</b>	mammary epithelial basal medium
<b>MEGM</b>	mammary epithelial cell growth medium
<b>MFP</b>	mammary fat pad
<b>mTORC1</b>	mammalian target of rapamycin complex 1
<b>NAAE</b>	non-adherent ALDH enriched
<b>NOD/SCID</b>	nonobese diabetic/severe combined immunodeficient
<b>OCT-4</b>	octamer-binding transcription factor 4
<b>PBS</b>	phosphate buffered saline
<b>ROS</b>	reactive oxygen specific
<b>STAT3</b>	signal transducer and activator of transcription 3
<b>SDS-PAGE</b>	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
<b>SE</b>	standard error
<b>SFRPs</b>	secreted frizzled-related proteins
<b>TCF</b>	T-cell transcription factor
<b>TDT</b>	tumor-doubling time
<b>TNBC</b>	triple negative breast cancer
<b>TOPflash</b>	TCF/LEF activity reporter
<b>TRAIL</b>	tumor necrosis factor-related apoptosis-inducing ligand

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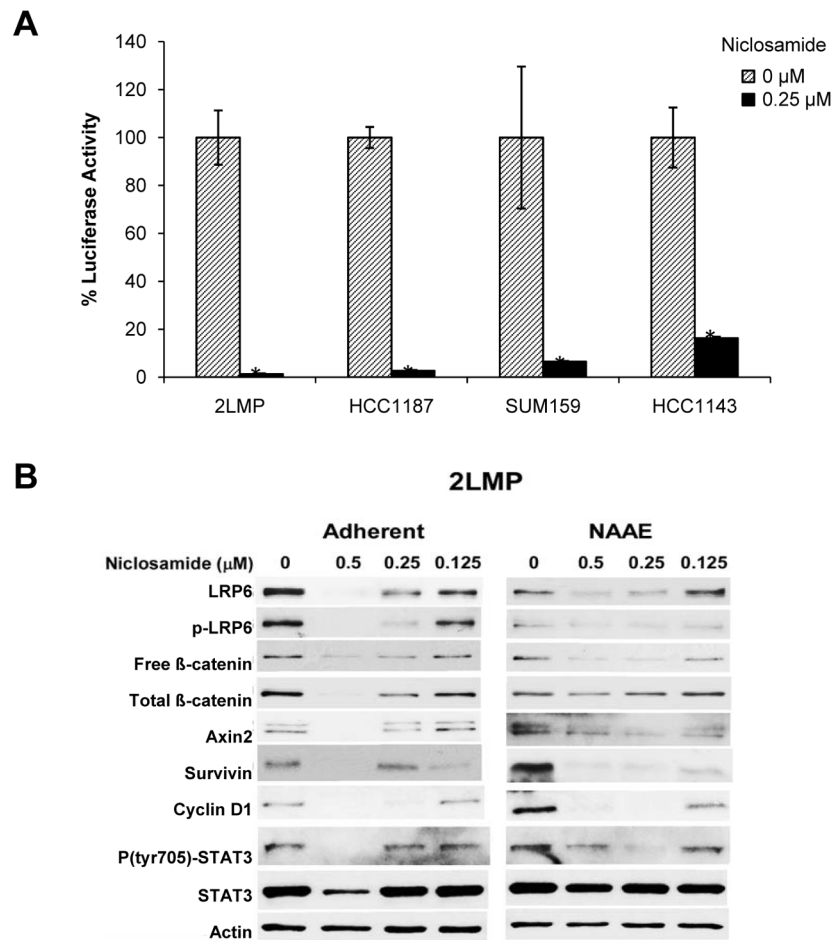
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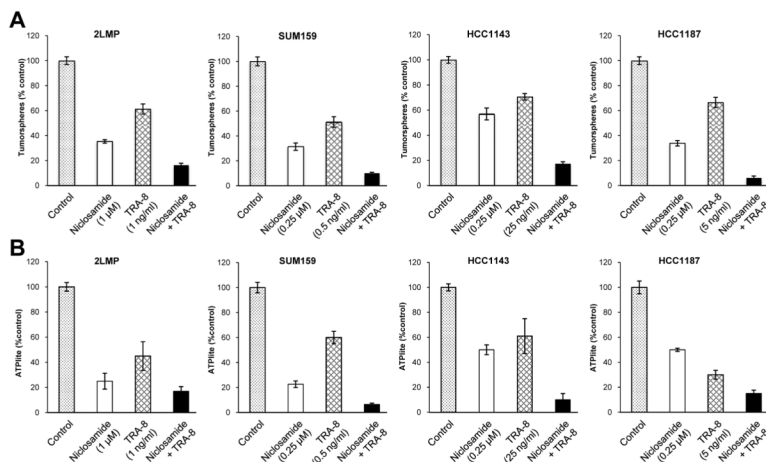
**Figure 1. Characterization of non-adherent ALDH enriched (NAAE) cells**

(A) 2LMP and SUM159 cells were FACS sorted for ALDH+ cells and analyzed for ALDH expression at 0, 6, 12, 24, 48, and 72 hours after sorting (solid line) and compared to unsorted cells in ultra-low attachment plates with serum free media (dotted line). HCC1143 cells were FACS sorted for ALDH+ cells and analyzed for ALDH expression at 0, 6, 12 and 24 hours. Unsorted cells harvested at the 12 hour time-point are called NAAE cells. (B) 20,000 2LMP NAAE cells (circle line) and 20,000 2LMP cells cultured in identical non-adherent (NA) conditions for 72 hours (square line) were injected into MFP of NOD/SCID mice and tumor growth was measured over time (difference at 48 days,  $P = 0.01$ ). (C) Adherent and NAAE 2LMP cells were analyzed by Western blot for expression of Wnt/ $\beta$ -catenin signaling proteins.



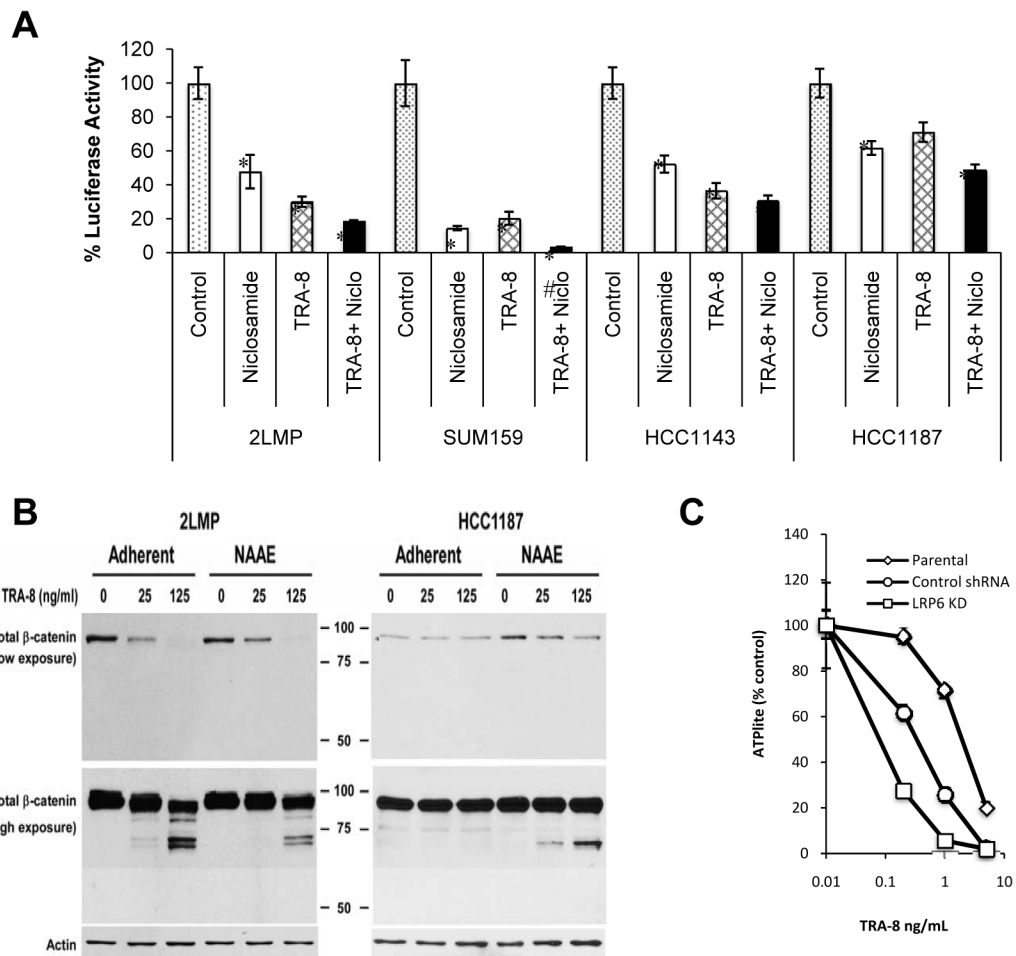
**Figure 2. Niclosamide effect on Wnt/ $\beta$ -catenin and STAT3 signaling**

(A) Activity of TCF/LEF reporter in the TOPflash assay was evaluated in 2LMP, HCC1187, SUM159 and HCC1143 NAAE cells treated with 0.25  $\mu\text{M}$  niclosamide for 24 hours. The experiment was performed in triplicate. The bars represent means  $\pm$  SE. Niclosamide treatment (solid black bars) was compared to untreated control cells (striped bars) (\* $P < 0.01$ ). (B) Western blot analysis of adherent and NAAE 2LMP cell lines after 24-hour treatment with niclosamide (0, 0.5, 0.25 and 0.125  $\mu\text{M}$ ).



**Figure 3. The effect of TRA-8, niclosamide and the combination on tumorsphere formation and cytotoxicity in NAAE cells**

(A) NAAE cells were cultured with media alone, pre-treated with niclosamide (0.25–1.0 μM) for 24 hours, followed by TRA-8 (1 ng/mL 2LMP, 0.5 ng/mL SUM159, 25 ng/mL HCC1143 and 5 ng/mL HCC1187) for 24 hours, for a total of 48 hours treatment. Tumorsphere formation was determined in 4 replicates and 3 separate experiments. ( $P < 0.05$  treatment vs. control,  $P < 0.05$  combination vs. either agent alone). Additive drug interaction against tumorsphere inhibition was observed for all four cell lines ( $P = 0.03$ ). (B) Cell viability of drug combination was also tested on 2LMP, SUM159, HCC1143 and HCC1187 NAAE cells. 2LMP and SUM159 cells were pre-treated for 24 hours with (0.25–1.0 μM) niclosamide, followed by TRA-8 (1 ng/mL 2LMP, 0.5 ng/mL SUM159 for 24 hours, for a total of 48 hours). HCC1143 and HCC1187 were treated concurrently with (0.25 μM) niclosamide, and TRA-8 (25 ng/mL HCC1143 and 5 ng/mL HCC1187), for a total of 48 hours treatment.

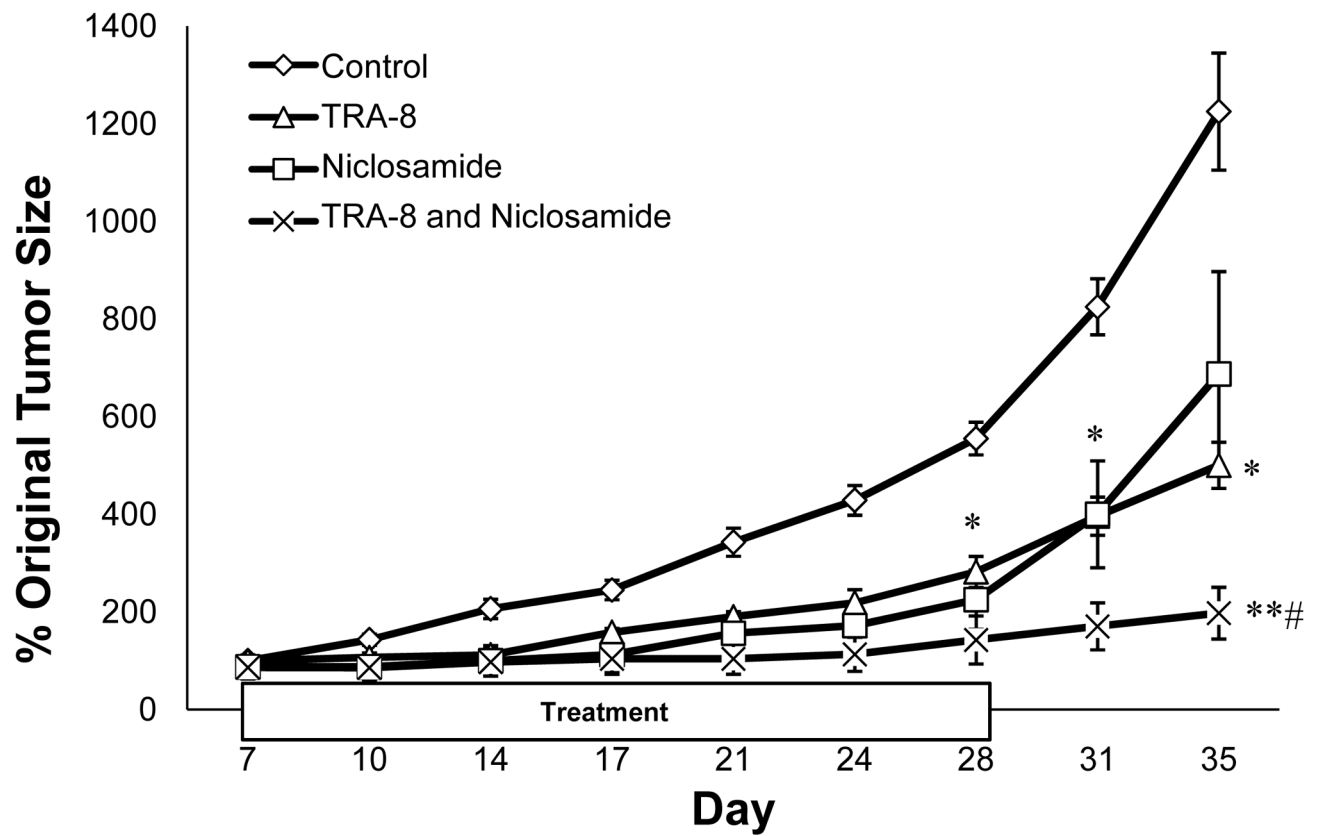


#### Figure 4. Niclosamide in combination with TRA-8 inhibits Wnt/β-catenin signaling

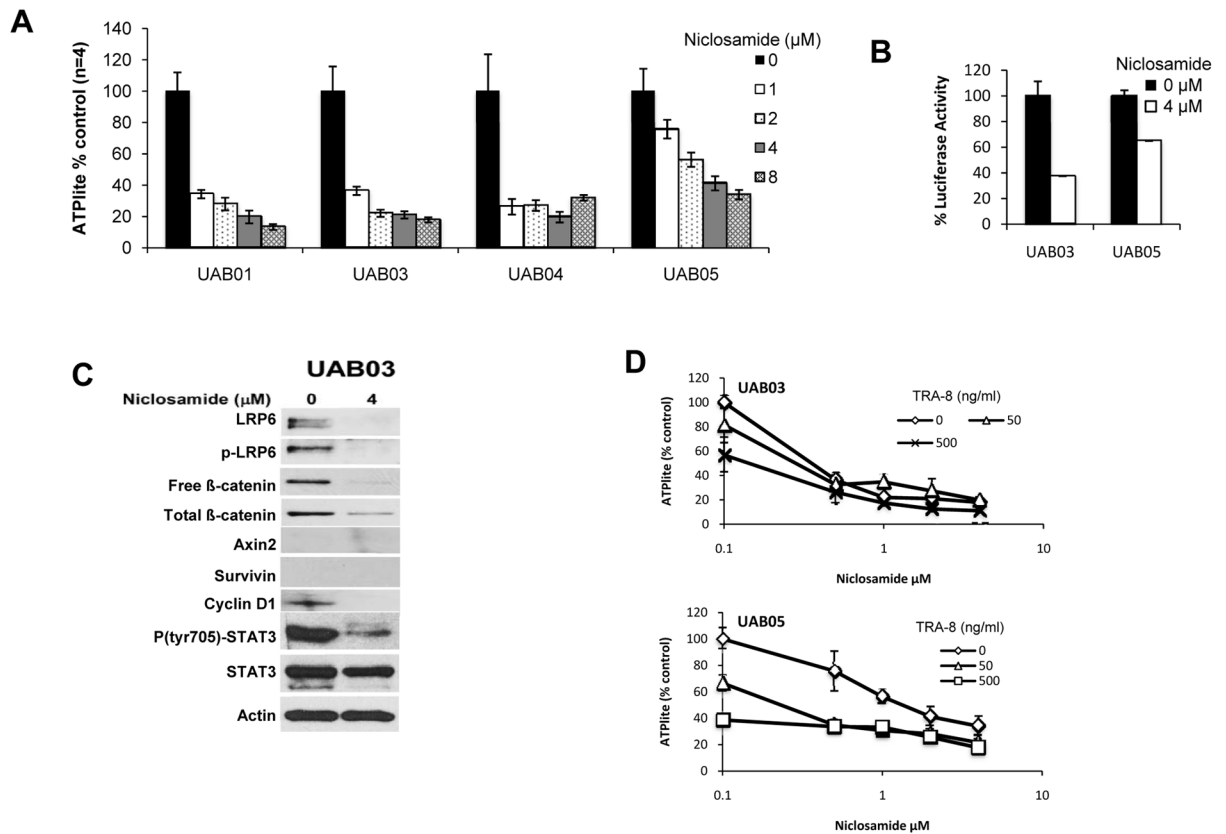
(A) Activity of TCF/LEF plasmid reporter in the TOPflash assay was evaluated in 2LMP, SUM159, HCC1143 and HCC1187 adherent cells. All cell lines were treated with 0.25 μM niclosamide for 48 hours and 0.25 ng/mL TRA-8 for 24 hours. The experiment was performed in triplicate. The bars represent mean ± SE. Single or combination treatment compared to control (\* $P < 0.05$ ), combination treatment vs. either single agent (# $P < 0.05$ ).

(B) Western blot analysis of β-catenin degradation was performed after 2-hour treatment with TRA-8 on both adherent and NAAE cell populations of the 2LMP and HCC1187 cell lines (top blot low exposure, bottom blot high exposure).

(C) 2LMP parental, LRP6 KD and shRNA control cells were treated with TRA-8 for 48 hours. The cytotoxicity was measured by ATPlite. The experiment was performed in triplicate. LRP6 KD compared to control shRNA ( $P = 0.01$ ).



**Figure 5. Effect of niclosamide and TRA-8 *in vivo* on 2LMP orthotopic tumor growth**  
 Tumors were established in athymic nude mice by MFP implantation of  $2 \times 10^6$  2LMP cells. The therapy started when tumors reached a size of  $16 \text{ mm}^2$ . Niclosamide (30 mg/kg) was given IP 5 days a week, TRA-8 (200  $\mu\text{g}$ ) was given IP 2 $\times$  weekly for 3 weeks. Tumor size was measured with calipers twice a week. Each point in the curve represents the mean  $\pm$  SE (n=5). Single agent niclosamide or TRA-8 vs. control (\* $P < 0.05$ ), combination treatment vs. control (\*\* $P < 0.01$ ), combination treatment vs. TRA-8 or niclosamide (# $P < 0.05$ ).



**Figure 6. Metastatic pleural effusion patient sample sensitivity to niclosamide alone and in combination with TRA-8**

(A) Sensitivity of patient samples to 48 hour niclosamide mediated cytotoxicity (1, 2, 4, and 8  $\mu\text{M}$ ) compared to untreated controls. Individual experiments were assayed in quadruplicate and bars represent mean  $\pm$  SE ( $P < 0.01$ ). (B) Activity of TCF/LEF viral reporter in the TOPflash assay was evaluated for patient samples UAB03 and UAB05. Both samples were treated with 4  $\mu\text{M}$  niclosamide for 24 hours in triplicate. The bars represent mean  $\pm$  SE. Niclosamide compared to control ( $*P < 0.05$ ). (C) Western blot analysis of patient sample UAB03 after 24 hour treatment with niclosamide (4  $\mu\text{M}$ ). (D) Cytotoxicity of patient samples UAB03 and UAB05 pre-treated with niclosamide (0.5, 1, 2, 4, and 8  $\mu\text{M}$ ) for 24 hours followed by 24 hours with TRA-8 (50 or 500 ng/ml). Cell viability was analyzed using ATPlite assay.