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Effects Of Novel Inhibitors Against Wnt/**B**-Catenin Signaling In Ovarian And Triple-Negative Breast Cancers

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EFFECTS OF NOVEL INHIBITORS AGAINST WNT/β-CATENIN SIGNALING IN OVARIAN AND TRIPLE-NEGATIVE BREAST CANCERS

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ABSTRACT

Ovarian and triple-negative breast cancers (TNBC) are aggressive due to their dependence on Wnt/β-catenin signaling. Niclosamide, a strong inhibitor of Wnt/β-catenin signaling, is an efficacious cytotoxic agent for various cancers *in vitro*, including ovarian and breast cancers. However, it has cross-reactivity with mTOR, STAT3, and Notch pathways. A limitation of using the agent in animal studies involves its poor water solubility and bioavailability. This study investigates novel compounds based on the structure of niclosamide to improve specificity for the Wnt/β-catenin signaling pathway. The investigation evaluates three questions: 1) whether WNT974 inhibits Wnt/β-catenin signaling in ovarian cancer; 2) whether novel analogs of niclosamide retain capacity to inhibit Wnt/β-catenin and mTOR signaling activities comparable to that of niclosamide in ovarian cancer; 3) and whether novel benzimidazole inhibitors developed at Southern Research are selective for Wnt/β-catenin signaling in TNBC over noncancerous cells.

Porcupine inhibitor WNT974 obtained from Novartis was evaluated as an agent with therapeutic efficacy against ovarian cancer. The agent inhibited Wnt/β-catenin signaling and promoted cell cycle arrest. Combination treatment of human ovarian cancer ascites cells with WNT974 and carboplatin yielded greater reduction in ATP levels than either drug alone. While Wnt/β-catenin-activating RSPO fusions were not present in the samples, porcupine expression correlated with sensitivity to WNT974.

Analogs 11 and 32 were developed by Dr. Pui-Kai Li at Ohio State University to improve on capacity of niclosamide to treat ovarian cancer. Analogs 11 and 32 inhibited proliferation of ovarian cancer cell lines and mouse patient-derived xenograft cells. Analogs 11 and 32 also inhibited Wnt/β-catenin and mTOR signaling pathways comparable to niclosamide.

The property of niclosamide to target multiple pathways, such as mTOR, STAT3, and Notch, is believed to confer toxicity in normal tissues and mitigate anti-tumor efficacy. To limit normal tissue toxicity and enhance efficacy in cancers, novel benzimidazole compounds developed at Southern Research were evaluated for their potency and Wnt/β-catenin-inhibiting efficacy in TNBC cell lines. Compounds SRI33576 and SRI35889 show specificity for Wnt/β-catenin signaling with limited effects on other pathways. These compounds exhibited a greater decrease in cell viability in TNBC cell lines than in nontumorigenic MCF10A cells *in vitro*.

Keywords: Ovarian Cancer, Triple-negative Breast Cancer, Wnt/β-catenin, Benzimidazole, LRP6, Targeted Therapy

DEDICATION

I dedicate this dissertation to my parents and brother for their unwavering support and to my late grandfather for his inspirational attitude towards learning.

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LIST OF ABBREVIATIONS

APC	Adenomatous Polyposis Coli
BSA	Bovine Serum Albumin
CSC	Cancer Stem Cell
DKK1	Dickkopf-1
DMSO	Dimethyl Sulfoxide
ECL	Enhanced Chemiluminescence
EMT	Epithelial-Mesenchymal Transition
EOC	Epithelial Ovarian Cancer
ER	Estrogen Receptor
FBS	Fetal Bovine Serum
FZD	Frizzled
HER-2	Human Epidermal Growth Factor-2
HGSOC	High-Grade Serous Ovarian Cancer
ICC	Immunocytochemistry
LRP6	Low-density Lipoprotein Receptor-Related Protein 6
mAb	Monoclonal Antibody
PBS	Phosphate-Buffered Saline
PDX	Patient-Derived Xenograft
PORCN	Porcupine

PR	Progesterone Receptor
RNF	Ring Finger
RSPO	R-spondin
SAR	Structure-Activity Relationship
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
sFRP	Secreted Frizzled-Related Protein
SR	Southern Research
TCF/LEF	T-cell factor/Lef-1 Family of Transcription Factors
TNBC	Triple-Negative Breast Cancer

INTRODUCTION

Ovarian Cancer

Ovarian cancer is the fifth worst cause of cancer-related death in women and is the leading cause of death due to gynecological cancer [1]. Majority of ovarian cancer patients exhibit stage III or IV disease [2]. Globally, less than 30% of women affected by advanced ovarian cancer survive 5 years following diagnosis. Epithelial ovarian cancers (EOCs) derive from the ovarian surface epithelium. More than 80% of all ovarian cancers are represented by EOCs. They normally attain poor differentiation from tissue of origin and consist of one of five histologic subtypes: serous, endometrioid, mucinous, clear cell, and undifferentiated. The serous subtype is the most prevalent [3].

Treatment of Ovarian Cancer

Patients with recurrence following at least 6 months after the last platinum treatment normally undergo further platinum-based therapy. Patients with recurrence following less than 6 months after the last platinum treatment receive single-agent chemotherapy, such as topotecan and gemcitabine [4]. Combination of paclitaxel and platinum as a therapeutic regimen has led to an improvement in overall survival for newly diagnosed patients. Neoadjuvant chemotherapy is an appealing alternative treatment, particularly for patients too weak to undergo surgery [5, 6].

Triple-Negative Breast Cancer

Triple-negative breast cancer (TNBC) is a subtype defined by the lack of estrogen receptor (ER), progesterone receptor (PR), and HER-2 amplification. TNBC accounts for about 15% of all breast cancers. TNBC patients exhibit higher frequency of metastatic disease recurrence within three years of diagnosis. Prognosis for patients with TNBC is poor compared to that for patients with other subtypes of breast cancer [7, 8].

Treatment of Triple-Negative Breast Cancer

Compared to other subtypes of breast cancer, TNBC is more resistant to traditional therapies such as hormone receptor-targeting agents and trastuzumab. As a result, TNBC is commonly treated with chemotherapy. While this subtype may show initial sensitivity to chemotherapies such as taxanes and doxorubicin, recurrence is observed within 3 to 5 years following treatment [9]. Poor prognosis associated with TNBC has been attributed to enrichment of tumor-initiating cells, or cancer stem cells (CSCs). Identifying therapies that target pathways mediating tumor initiation is an appealing strategy to prevent recurrence and metastasis in patients [10].

Wnt/β-catenin Signaling

The Wnt/β-catenin pathway plays a critical role in normal embryonic development and growth of organisms and has been shown to participate in the development of various organs. Signaling is activated by secreted Wnt glycoproteins with the capacity to transduce signaling by binding to transmembrane receptors. Ligands, such as Wnt1, -3a, -8, -8a, are categorized in a proto-oncogenic group which activates

canonical β-catenin signaling. Another group is known to activate noncanonical signaling and to inhibit function of proto-oncogenic Wnt ligands [11].

Encoded by the CTNNB1 gene, β-catenin on the intracellular side is involved in cellular adhesion, connecting cadherins to actin filaments. Wnt/β-catenin signaling is initiated by Wnt ligand binding to LRP5/6 and Frizzled (FZD) co-receptors. This leads to stabilization of cytoplasmic β-catenin after its accumulation and localization in the nucleus, where it binds to TCF/LEF1 and enables transcription of specific genes, such as cyclin D1, survivin, and c-myc. Without initiation of Wnt/β-catenin signaling, β-catenin is bound by a destruction complex composed of adenomatous polyposis coli (APC), Axin, and glycogen synthetase kinase-3β. This complex phosphorylates β-catenin, leading to its subsequent ubiquitination and proteasomal degradation (as depicted in Figure 1).

Wnt/β-catenin Signaling in Ovarian Cancer

Investigations have revealed upregulated expression of nuclear β-catenin in ovarian cancers, associated with poor prognosis in patients. Nuclear accumulation of β-catenin is implicated in cell migration, cancer stem cell renewal, and chemoresistance. One study found 23% of high-grade serous ovarian cancers (HGSOCs) exhibited nuclear β-catenin expression compared to only 2.1% for low-grade serous carcinomas [12]. Dysregulation of Wnt/β-catenin signaling may be attributed to mutations of CTNNB1, which has been observed primarily in endometrioid subtype of ovarian cancer, as these mutations have been associated with upregulated nuclear β-catenin expression [3, 13].

Wu, et al. identified such presence in 12 of 45 (26.7%) endometrioid tumors [14]. Metastatic lesions exhibit higher levels of β-catenin compared to primary epithelial ovarian carcinomas [15]. Significant increase in β-catenin and GSK3β was detected in adenocarcinomas compared to normal ovarian tissue and benign adenomas. Decrease in APC expression has been observed in ovarian adenocarcinomas, and Lef-1 was maintained throughout all analyzed tissues [16]. In addition, constitutive β-catenin/Lef-1-mediated transcriptional activity was observed in 21% of evaluated ovarian cancer cell lines [17]. Knockdown of β-catenin sensitized A2780 ovarian cancer cells to cisplatin, paclitaxel, and vincristine [18].

While nuclear β-catenin accumulation is commonly observed in epithelial ovarian cancer, genetic mutations in components of the Wnt/β-catenin pathway are rare [15]. Rather, Wnt/β-catenin induction appears to initiate via Wnt ligand-receptor interaction because of overexpression of ligands and receptors and downregulated presence of secreted inhibitors. Wnt7a regulates normal cellular and developmental pathways implicated in prenatal growth of female reproductive tract and maintaining uterine function in rodents [19]. WNT7A is overexpressed in ovarian epithelial cells and functions via FZD in the mesenchyme and epithelium. One study found WNT7A is not expressed in normal adult ovarian tissues but is overexpressed in many malignant ovarian tumors. Repression of WNT7A in ovarian cancer cell line SKOV3.ip1 reduced tumor formation and metastasis in the peritoneal cavity. WNT7A was also found to regulate proliferation, adhesion, invasion, and cell cycle progression of ovarian cancer cells. In addition, Wnt/β-catenin transcriptional targets MMP7 and cyclin D1 were increased [20]. Other components such as the Wnt antagonist SFRP1 gene, located on chromosome

8p11.2, is frequently silenced by methylation in ovarian cancer [21]. In addition, the SFRP1 gene may not be expressed due to chromosomal loss of 8p11.2 [22, 23]. Secreted R-spondin (RSPO) proteins induce Wnt/β-catenin signaling, and RSPO mutations are associated with upregulated Wnt/β-catenin signaling [24]. RSPO1 amplifications have have been observed in 8% of epithelial ovarian cancer [25, 26].

Wnt ligands undergo lipidation via addition of palmitoyl group to a conserved serine by membrane-bound O-acyltransferase Porcupine [27]. Lipidation of Wnt is required for secretion from the endoplasmic reticulum [28] and necessary for Wnt functionality. Porcupine (PORCN) is an enzyme which functions in Wnt ligand modification prior to secretion [29]. WNT974 selectively inhibits PORCN activity and decreases Wnt ligand secretion into the extracellular environment, thus attenuating cell surface receptor activation and subsequent Wnt/β-catenin signaling. The agent suppresses growth of Wnt-dependent tumors, including pancreatic and head and neck squamous cell cancers containing mutations in Wnt pathway mediators such as Ring finger 43 (RNF43) [30].

Wnt/β-catenin Signaling in TNBC

Wnt/β-catenin signaling causes cell proliferation and is upregulated in TNBC compared to other breast cancer subtypes. Like ovarian cancer, proliferation of TNBC is driven by autocrine Wnt/β-catenin signaling [31]. Also, like ovarian cancer, TNBC rarely displays mutations of Wnt/β-catenin signaling, and therefore, high activity is associated with dysregulated expression of certain components. Negative regulators such as secreted FZD-related proteins (sFRPs) and Dickkopf 1 (DKK1) show low expression in TNBC.

A study of 245 invasive breast carcinomas revealed the TNBC subtype to have accumulated nuclear β-catenin. In addition, no β-catenin mutations were identified in TNBC samples analyzed [32]. One study reported higher prevalence of cytoplasmic and nuclear β-catenin in basal-like breast cancers compared to other subtypes. It was also found that cytoplasmic and nuclear β-catenin expression in basal-like breast cancers was associated with phenotype of CD44+/CD24- stem cells [33].

LRP6 is overexpressed in triple-negative, ER-negative, and HER2-negative breast cancers. At the genetic level, LRP6 expression was more than 6-fold higher in 6 of 7 TNBC cell lines compared to noncancerous MCF10A cells [34]. At the protein level, LRP6 was overexpressed in 4 of 7 TNBC cell lines compared to MCF10A cells. Little overlap was observed between tumor samples displaying higher LRP6 transcript and those with higher HER2 transcript, signifying that Wnt/β-catenin signaling is possibly an independent prognostic indicator. Inhibition of LRP6 expression and/or activity with Mesd, an LRP6 antagonist, in MDA-MB-231 TNBC cells decreased cell viability, proliferation, and colony formation [34]. In addition, inhibition of LRP6 activity led to a decrease in migration and invasion of TNBC cells [35]. Treatment with Mesd also decreased expression of S100A4, a Wnt/β-catenin signaling target and a contributor to cancer metastasis. As a result, targeting LRP6 represents an appealing therapeutic strategy for TNBC (as depicted in Figure 1).

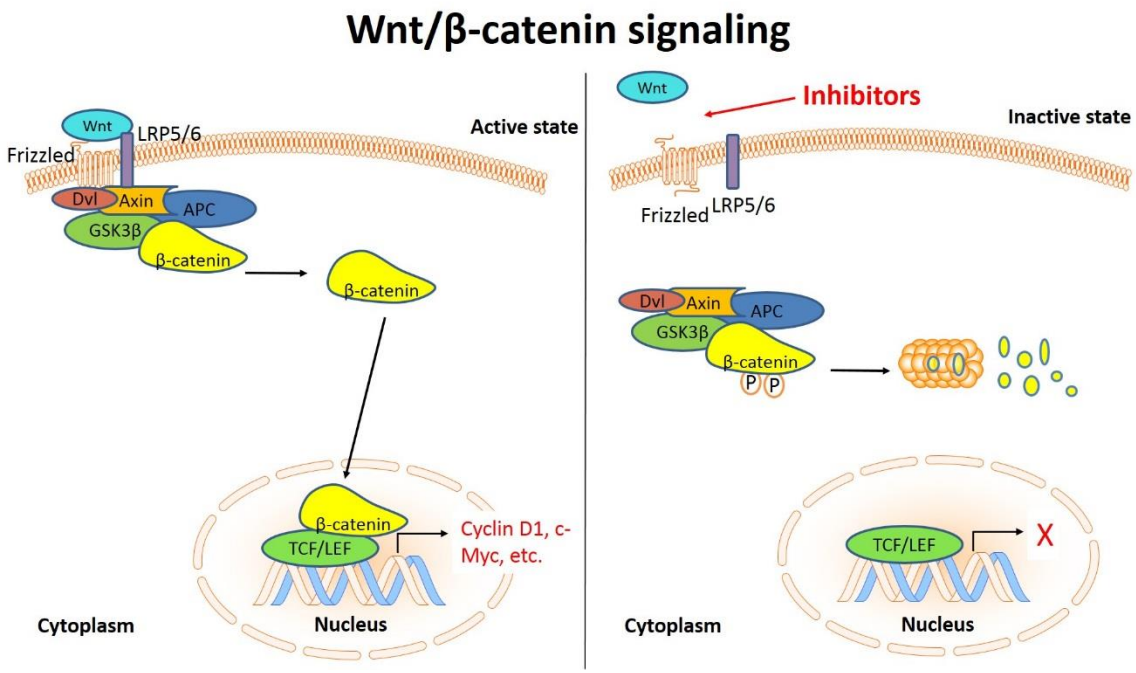


Figure 1. Wnt/β-catenin signaling. In the active state, Wnt binding to co-receptors Frizzled and LRP5/6 leads to inhibition of phosphorylation of β-catenin. Free β-catenin translocates to the nucleus and binds to TCF/LEF, promoting transcription of pro-tumorigenic genes (cyclin D1, c-Myc, survivin, etc.). In the inactive state, Wnt does not bind to the co-receptors, leading to phosphorylation (via GSK3β), ubiquitination, and proteasomal degradation of β-catenin. Degradation, prevents free β-catenin from translocating into the nucleus to promote transcription. Inhibitors targeting the interaction of Wnt ligands with the co-receptors represent appealing therapies in cancers.

Wnt pathway genes, such as LRP6, TCF7, and FZD7, were identified as significantly overexpressed in TNBC [36, 37]. FZD7 was identified as the only Frizzled family member significantly overexpressed in TNBC. Axin, GSK3, and DVL genes did not show significant change in expression. These proteins may be active via phosphorylation-induced conformational change or mutations [37]. Evidence suggests inhibition of either FZD7 or LRP6 will obstruct Wnt/β-catenin signaling and mitigate Wnt/β-catenin pathway-regulated cell proliferation [37, 38]. LRP6 and Wnt target gene SOX9 regulate each other in TNBC cell lines. LRP6 overexpression leads to SOX9 increase, while knockdown of SOX9 decreased LRP6 transcription and lowered cell invasion and proliferation [39]. LRP6 overexpression correlates with increased Wnt/β-

catenin signaling and higher stemness in TNBC cells [40]. Syndecan-1 is an epithelial-mesenchymal (EMT) marker linked with development and breast tumorigenesis, implicated in regulating TNBC stem cell properties by increasing LRP6 expression and Wnt/β-catenin signaling [40]. Breast cancer cell lines expressing high HER2 levels typically exhibited low FZD7 levels, highlighting the importance of the receptor in TNBC.

The 8p11 region also undergoes chromosomal breaks, loss, and amplification in breast cancer. Amplification occurs in approximately 15% of breast cancers. The region is linked to disease-related survival and metastatic recurrence in breast cancer patients [41-44]. One study found the region of recurrent amplification at 8p11-12 was decreased in copy number in some breast cancers and this was linked to poor prognosis [45]. SFRP1 was identified as an under-expressed gene despite its increase in copy number [46]. SFRP1 mRNA expression was identified as lost in more than 80% of invasive breast cancers [47]. One study showed SFRP1 obstructs proliferation of numerous breast cancer cell lines via disruption of pathway induction mediated by endogenous WNT ligands [48]. Methylation of SFRP1 promoter was observed in 61% of primary breast cancers. In addition, SFRP1 gene hypermethylation was linked to shorter survival of invasive breast cancer patients [49].

Treatment of TNBC with docetaxel or doxorubicin, while ineffective on growth of cell lines, correlated with deregulation of genes linked to stemness in TNBC cells [50]. Doxorubicin treatment led to deregulation of stem cell signaling pathways linked to cell growth, renewal, and differentiation. Expression of genes encoding Wnt signaling components, such as FZD2, FZD4, FZD5, FZD6, FZD7, FZD9, Wnt5a, Wnt10a, Wnt11,

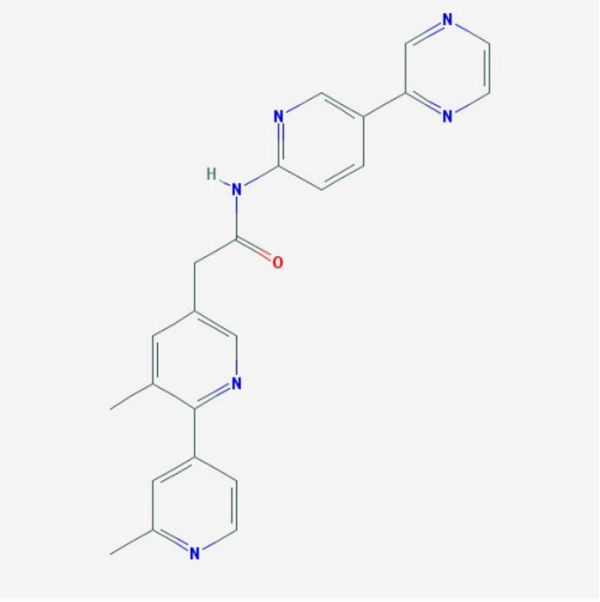
and Axin was affected by treatment. The study showed docetaxel and doxorubicin treatment induce stemness in TNBC cells, thus conferring acquired chemoresistance in refractory TNBC [50].

Wnt/β-catenin Inhibitors as Therapies for Cancer

Wnt/β-catenin signaling is implicated in various developmental processes and maintenance of tissue homeostasis. It is understood inhibition of overall Wnt/β-catenin signaling is unsafe due to broad expression of Wnt ligands and LRP5/6/FZD co-receptors and involvement of the pathway in biological processes, such as differentiated epithelium homeostasis, maintenance of pluripotent stem cells, and bone homeostasis. A major hurdle in effective TNBC treatment with Wnt/β-catenin inhibitors is toxicity of normal tissues. Agents have been developed with limited efficacy against TNBC due to their interaction with non-Wnt/β-catenin pathways. Inhibition of Wnt/β-catenin signaling by expression of Dkk1, an LRP6 antagonist, or loss of TCF4, abrogated epithelial proliferation in small intestine and colon in mice and led to loss of crypts, villi, and glandular structure within a week [51, 52]. Wnt/β-catenin inhibitors such as tankyrase inhibitors were also found to elicit intestinal toxicity [53, 54]. Ectopic expression of Dkk1 was associated with total failure of hair follicle formation in adult mice [55]. In opposition, *in vivo* treatment with LRP6 antagonist obstructed MMTV-Wnt1 tumor growth while not causing unwanted side effects [34].

Thus far, studies investigating Wnt/β-catenin inhibitors have shown more promise *in vitro* than *in vivo*. C59, an inhibitor of Wnt ligand modification and subsequent secretion, did not significantly inhibit cancer cell proliferation in 46 tested cell lines,

suggesting Wnt secretion is not a major contributor to tumor growth. Some cell lines showed inhibition of proliferation at more than 1.5 μM, or 20,000 times greater than the IC_{50} value [56]. This effect is likely a consequence of off-target effects in certain cell lines. In addition, tumors from mice treated with C59 contained normal staining of membrane β-catenin and significantly decreased cytoplasmic and nuclear β-catenin. At the administered dose, C59 was concluded not to elicit toxicity in normal tissues as the treated mice did not exhibit loss of body weight. In addition, several tissues examined histologically did not show indications of toxicity. The treated mice showed normal intestinal morphology [56]. Another inhibitor of Wnt secretion, LGK974 (renamed WNT974), required a high daily dose of 20 mg/kg but elicited lesser toxicity at effective doses [57]. Continuous Wnt/β-catenin signaling inhibition by WNT974 was found not to be obligatory for tumor regression, presenting a therapeutic window for drug activity while sparing normal tissues.



WNT974

Most anti-cancer agents, including small molecule inhibitors and monoclonal antibodies (mAbs), target highly proliferative cells which constitute bulk cells but not CSCs. Trastuzumab targets the HER2 receptor which is highly expressed in 25% of breast cancers. However, administration of the single agent only yielded a 30% response rate and acquired resistance to trastuzumab emerges frequently. Self-renewal of CSCs is promoted by induction of embryonic Wnt/β-catenin, Notch, mTOR, and STAT3 signaling (Figure 2) [58, 59]. Transcriptional suppression of LRP6 or FZD7 co-receptors in TNBC inhibits tumor growth *in vivo* [37, 60]. A screening study identified salinomycin as a suppressor of LRP6 expression and Wnt/β-catenin signaling. In addition, it was found to be a selective therapeutic agent for breast CSCs [61, 62].

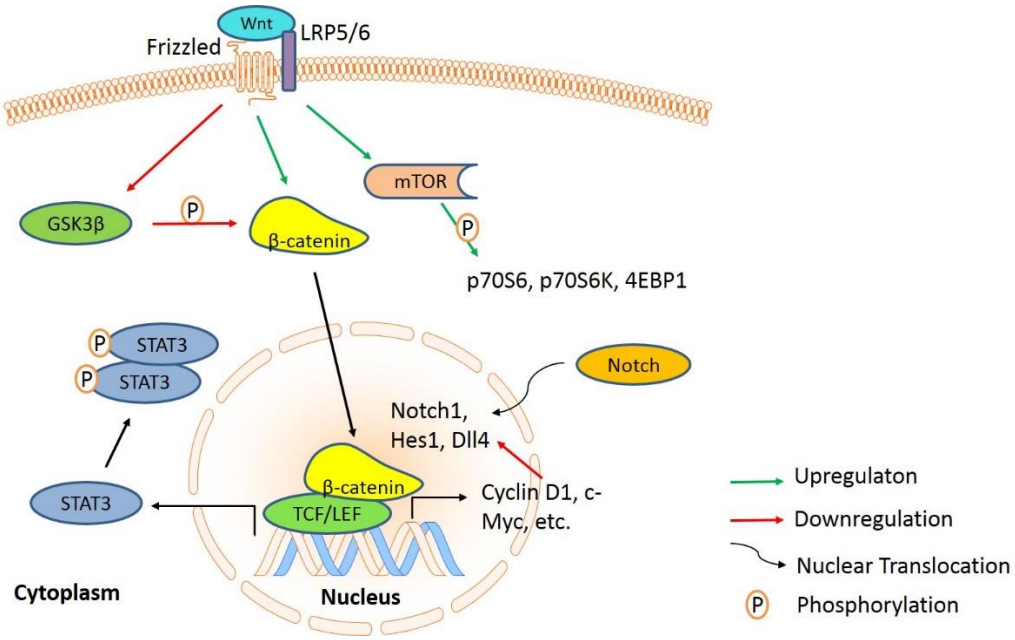


Figure 2. Interactions of Wnt/β-catenin, mTOR, STAT3, and Notch pathways. Numerous interactions are involved with the Wnt/β-catenin pathway. Proteins encoded by Wnt target genes (cyclin D1, c-Myc, survivin, etc.) inhibit transcription mediated by Notch. Wnt ligand binding to co-receptors has been shown to promote activation of p70S6, p70S6K, and 4EBP1 via mTOR. β-catenin-mediated transcription has been found to lead to STAT3 transcription followed by STAT3 accumulation in cytoplasm.

Although side effects have not been detected during many preclinical studies, they have emerged during clinical trials. Clinical trials for agents such as Oncomed Pharamaceuticals' Wnt/β-catenin inhibitors, vantictumab and OMP54F28, have been put on hold due to "mild to moderate" side effects involving the bones (http://www.reuters.com/article/2014/06/13/us-oncomed-study-idUSKBN0EO1B920140613). Such effects were observed in 8 out of 63 (13%) patients treated with vantictumab and 2 out of 41 (5%) individuals with OMP-54F28. One of the effects of vantictumab involves an increased bone turnover, thus it is possible the patients developed osteoporosis. Lessening the dosage and administration frequency of both agents could perhaps mitigate the side effects; however, drug effectiveness may also be compromised. Vantictumab has binding affinity for 5 out of 10 FZD family members *in vitro* and possibly more *in vivo*. In addition, not all FZDs binding to vantictumab are upregulated in solid tumors. Some FZD receptors may be integral toward bone remodeling. Agents with a lesser range of specificity may be effective, inhibiting FZDs implicated in tumorigenesis but not FZDs that play a role in osteogenesis. Safety in targeting the Wnt/β-catenin pathway may be attained by inhibiting a specific sub-pathway, such as β-catenin and TCF/LEF interaction, and not mitigating many sub-pathways altogether [63, 64]. In addition, Wnt/β-catenin signaling plays a major role in mediating resistance of HGSOC to platinum-based therapies [65]. An appealing therapeutic regimen involves combination of Wnt/β-catenin inhibitors with standard chemotherapeutic agents.

Studies with Niclosamide

Niclosamide is an FDA-approved anthelmintic agent administered for treatment of tapeworm infections. Niclosamide (5-chloro-4-N-(2-chloro- 4-nitrophenyl)-2-hydroxybenzamide) is provided by oral ingestion. A member of the salicylanilide class of anthelmintic drugs, niclosamide has also shown efficacy in preclinical studies against a variety of cancers including colon [66, 67], myeloma [68], lung [69, 70], and glioblastoma [71]. Our laboratory has previously shown niclosamide is cytotoxic against breast and ovarian cancer cell lines [72, 73]. Currently, the agent is undergoing clinical trials for treatment of prostate and colorectal cancers. A high throughput screening study revealed niclosamide as an efficacious Wnt/β-catenin inhibitor in colon CSCs [74]. Another study investigating 1200 FDA-approved drug and drug-like agents identified niclosamide as a robust instigator of FZD1 internalization [75].

Niclosamide has shown evidence of eliciting inhibitory effects against Wnt/β-catenin signaling by internalizing LRP6 and FZD1, leading to lessened signaling initiation [75, 76]. Niclosamide has also been identified as an inhibitor of other tumor-promoting signaling pathways such as mTOR[77], STAT3[70, 78], and Notch[67]. The agent promotes autophagy by inhibiting mTOR signaling activation, attenuating mitochondrial oxidative phosphorylation, disrupting mitochondrial membrane potential, and activating caspase-3.

Osada, et al. showed niclosamide concentrations within the plasma of mice were like those of the tumor, indicating effectual distribution of niclosamide from blood to tumor. Plasma concentrations of niclosamide were found to be stable at 39.5 to 77.6 ng/mL between 0.5 and 12 hours following oral administration of 200 mg/kg of the agent.

However, a major challenge in administering niclosamide for treatment of cancer involves its poor absorption after oral intake. High doses of oral-ingested niclosamide at 100-200 mg/kg body weight were required to observe significant inhibition of tumor growth in mice.

Niclosamide Analogs

Investigations have revealed mechanisms and potentially targetable targets within the Wnt/β-catenin pathway [75, 79-82]. Structure Activity Relationship (SAR) studies have identified inhibition of Wnt/β-catenin signaling by niclosamide as characteristically distinct among structurally similar salicylanilide class of anthelmintic agents evaluated. The studies also concluded Wnt/β-catenin signaling response relied on minor modifications of the chemical structure of niclosamide [75]. SAR has been studied to generate derivatives of niclosamide based on the salicylanilide structure. Inhibition of Wnt/β-catenin signaling activity was revealed to be based on type and location of substitution [83]. In addition, replacement of the nitro group, a major target for metabolism, with a chloro or trifluoromethyl substituent retained Wnt/β-catenin inhibitory efficacy. Significant loss of inhibitory response to Wnt3a stimulation was observed with replacement of the nitro group with an amine ($-NH_2$) or an acetamide ($-C_2H_5NO$) [79]. Certain changes in structure were also found to increase plasma concentration and longevity of exposure of compound. DK-520, a compound with a structure similar to analog 32, was a derivative discovered to increase plasma concentration and exposure duration *in vivo* while not eliciting toxic effects over three weeks of dosing. The agent exhibited comparable Wnt/β-catenin inhibitory efficacy and

inhibition of proliferation. DK-520 metabolizes to niclosamide, enabling greater exposure to niclosamide following oral administration.

This provided the impetus for us to evaluate other agents with similar structures as niclosamide to improve bioavailability based on the salicylanilide scaffold (Figure 3).

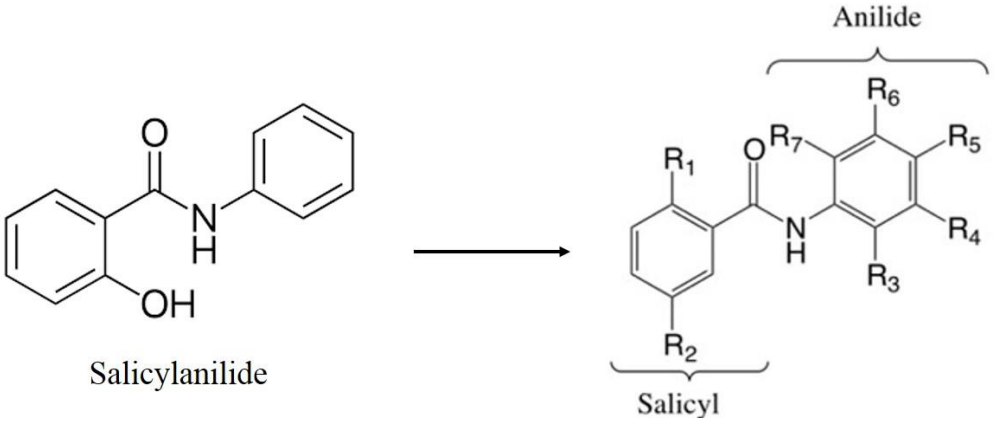


Figure 3. Generation of scaffold for niclosamide analogs based on the salicylanilide structure.

Replacement of the nitro group in analog 11 with electron-withdrawing group trifluoromethyl (CF3) provides enhanced stability and Wnt-inhibitory efficacy [83]. Analog 32 is the prodrug of niclosamide, an effort to improve on the bioavailability of niclosamide (Figure 4). Both analogs were found to downregulate Wnt/β-catenin signaling in human ovarian cancer patient ascites samples to a comparable extent as niclosamide (Figure 5).

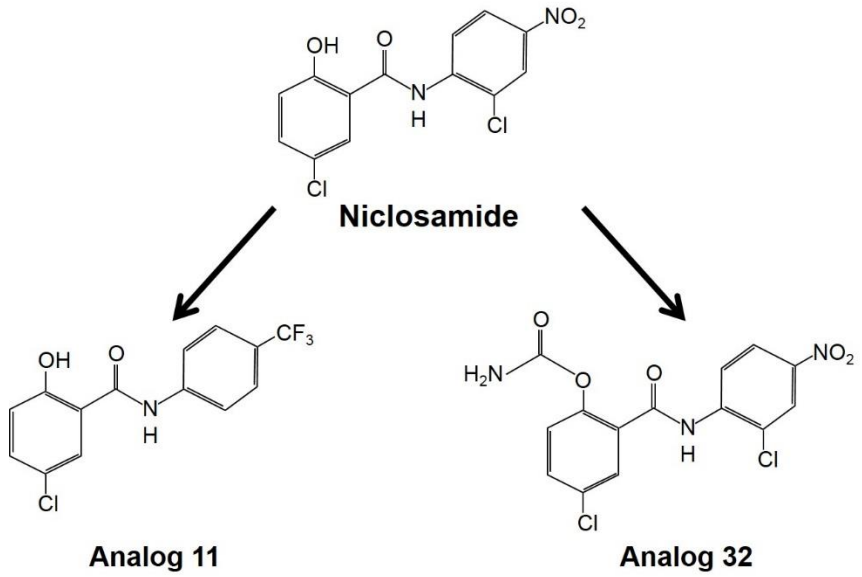


Figure 4. Structures of niclosamide, analog 11, and analog 32.

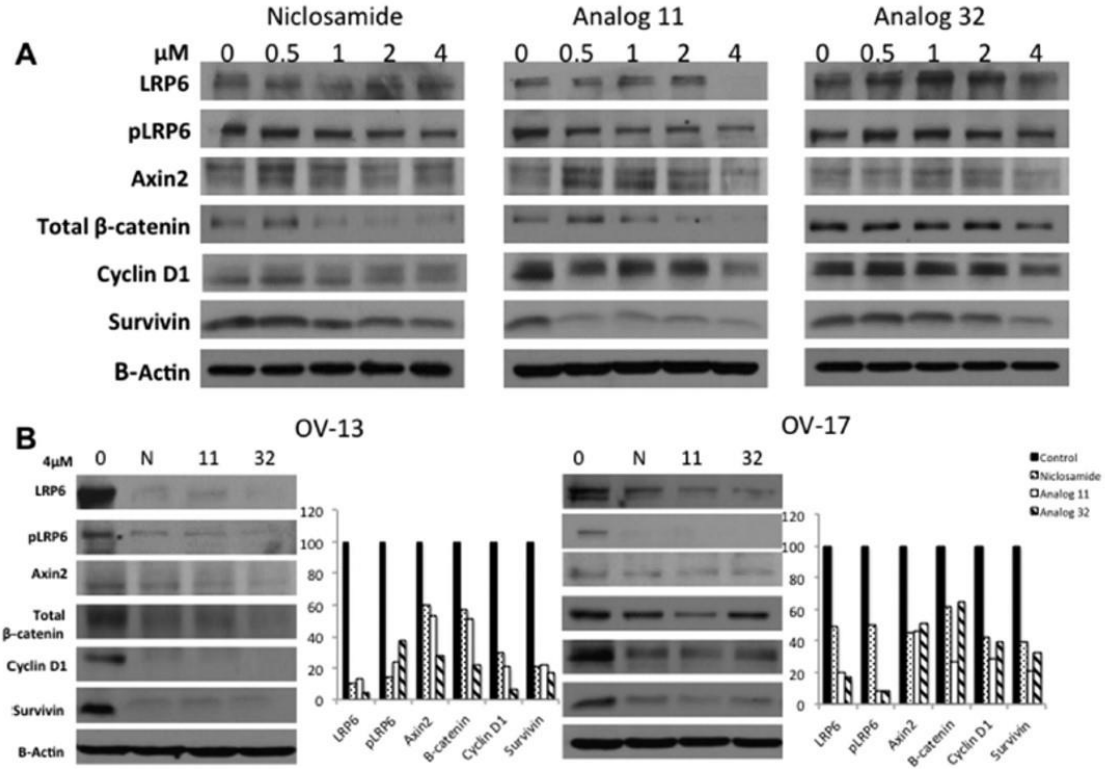
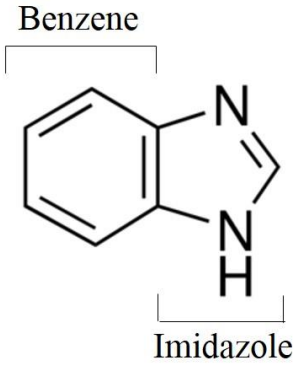


Figure 5. Inhibition of Wnt/β-catenin signaling by niclosamide and analogs 11 and 32 in patient ascites cells. A. Tumor cells isolated from a sample of patient ascites (OV-12) were treated with niclosamide, analog 11, and analog 32 (0.5-4 μM) for 24 hours. B. Tumor cells isolated from patient sample ascites (OV-13 and OV-17) were treated with niclosamide, analog 11, and analog 32 (4 μM) for 24 hours. Expression of Wnt/β-catenin pathway proteins was determined by Western blot analysis. Densitometry to determine

the decrease in expression of proteins is shown as percent of untreated control for the 4 μM-treated samples. From "Niclosamide analogs for treatment of ovarian cancer" by C.L. Walters Haygood, et al., 2015, *International Journal of Gynecological Cancer*, *25*, p. 1384. Copyright 2015 by IGCS and ESGO. Reprinted with permission.

Benzimidazoles

Benzimidazoles are compounds containing fused benzene and imidazole rings.



Benzimidazole derivatives have been found to elicit antioxidant [84], antiviral [85], antimicrobial [86], and antitumorigenic [87-93] effects. Their anticancer effects may be mediated by attenuation of poly (ADP-ribose) polymerase-1 (PARP-1) [87], topoisomerase I [88], cell cycle checkpoint kinase 2 [89], and tyrosine kinases [90, 91]. Compound 2-chloro-N-(2-p-tolyl-1H-benzo[d]imidazol-5-yl) acetamide, known as compound 2a, was identified as an efficacious inhibitor of breast tumor growth in animal studies via HER2 and EGFR inhibition and c-Jun N-terminal kinase (JNK)-mediated death receptor 5 upregulation. Derivative (S)-2-(2-fluoro-4-(pyrrolidin-2-yl) phenyl)-1H-benzimidazole-4-carboxamide, also known as compound 22b, was discovered to have sufficient *in vivo* efficacy in a breast cancer xenograft model both as a single agent and in combination with carboplatin. The agent also displayed adequate oral bioavailability with the capacity to effectively cross the blood-brain barrier and to also disseminate in tumor tissue [92].

Benzimidazoles have been evaluated as Wnt/β-catenin inhibitors. NCB-0846, an orally available inhibitor of TNIK, a regulator of Wnt/β-catenin signaling, was discovered as a benzimidazole with Wnt-inhibiting activity in colorectal cancer. The agent was found to target CSCs and inhibit tumorigenesis [93]. Mook, et al. evaluated Wnt/β-catenin-inhibiting efficacy of benzimidazole derivatives in colorectal cancer cells [94]. Niclosamide's therapeutic effects may be linked to its capacity to affect cellular ATP levels and uncouple oxidative phosphorylation in addition to its tendency to target multiple pathways. Benzimidazole compounds were identified with less effects on ATP homeostasis than niclosamide and were thus supported as agents with greater selectivity against Wnt/β-catenin signaling. However, effects of the compounds on pathways such as mTOR, STAT3, and Notch were not evaluated. Studies have shown inhibition of these pathways can increase Wnt/β-catenin signaling and could promote chemoresistance [95]. Despite identification of salinomycin as a selective agent for breast CSCs and an inhibitor of metastasis via targeting Wnt, Hedgehog, and STAT3 pathways [61], it did not affect growth of primary lung tumors. Salinomycin has not advanced through clinical studies possibly as a result of its apparent toxicity [96-99]. As a result, it is believed targeting a single pathway may elicit little toxicity due to specificity [100].

The benzimidazole scaffold was derived from constraining the amide bond of niclosamide. The 2,5-disubstituted benzimidazole compounds were developed by varying the substituents, such as the nitro and hydroxyl groups on the phenyl rings (Figure 6A). Formation of the ring in a benzimidazole is believed to add rigidity to niclosamide (salicylanilide) scaffold, affecting potency and drug-like properties of the compound while enhancing specificity. Based on the 2,5-disubstituted benzimidazole scaffold, five

compounds (SRI 32529, SRI 33576, SRI 35357, SRI 35361, and SRI 35889) were generated for evaluation of Wnt/β-catenin signaling selectivity (Figure 6B).

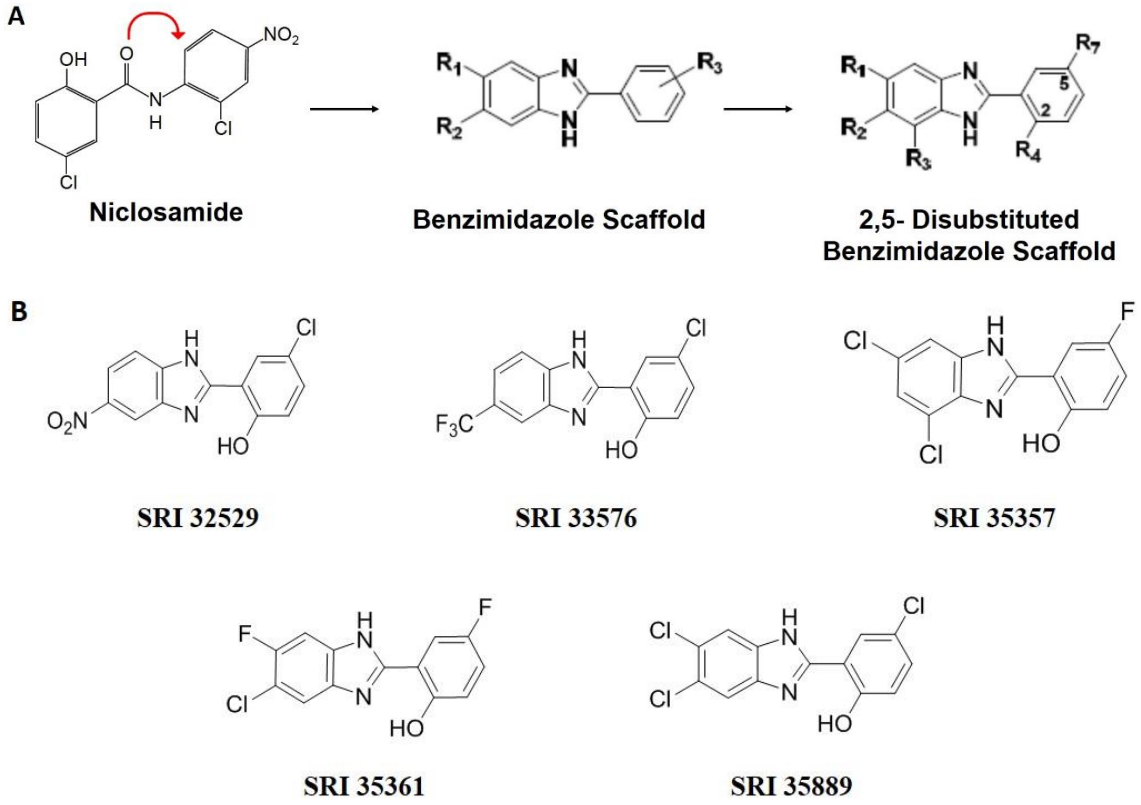


Figure 6. 2,5- disubstituted benzimidazole compounds (A) Schematic depicting constraining of amide bond (red arrow) in niclosamide to generate the benzimidazole scaffold. Varying substituents in the 2 and 5 positions of the phenyl ring generates 2,5-disubstituted benzimidazole compounds. (B) Compounds evaluated for Wnt/β-catenin signaling selectivity based on the 2,5-disubstituted benzimidazole scaffold.

Summary and Objective

I propose to investigate Wnt/β-catenin signaling selectivity and efficacy of novel compounds in ovarian cancer and TNBC. The results in the first study of this dissertation will exhibit antiproliferative and Wnt/β-catenin-inhibiting effects of WNT974 in primary ovarian cancer ascites. The second study shows cytotoxic effects of novel salicylanilide analogs of niclosamide in ovarian cancer. We show that these analogs of niclosamide inhibit proliferation and Wnt/β-catenin signaling activity in ovarian cancer cell lines and

primary ovarian cancer patient ascites samples, a few of which display platinum resistance. The third study will show effectiveness of treatment of TNBC cell lines with novel benzimidazole compounds that inhibit Wnt/β-catenin signaling without affecting the mTOR, STAT3, and Notch pathways. Two compounds were identified with higher specificity for Wnt/β-catenin signaling than niclosamide and low toxicity in noncancerous MCF10A cells.

While WNT974, niclosamide analogs, and the benzimidazole compounds have been evaluated in ovarian cancer and TNBC, these agents can be investigated in various other Wnt/β-catenin-dependent cancers, such as colorectal and pancreatic. The investigated inhibitors may also be combined with common chemotherapies to improve efficacy. The agents used in the studies have been evaluated *in vitro,* but further efforts to identify therapeutic efficacy will require *in vivo* studies in mouse models.

TARGETING THE WNT/β-CATENIN PATHWAY IN PRIMARY OVARIAN CANCER WITH THE PORCUPINE INHIBITOR WNT974

by

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Laboratory Investigation

ABSTRACT

More women in the United States die from ovarian cancer than any other gynecologic malignancy. It is estimated that there will be 21,290 new cases of ovarian cancer, and 14,180 women will die of the disease in 2015.(1) Over the last 20 years, advances using platinum/taxane doublets, dose-dense chemotherapy, intraperitoneal chemotherapy, and targeted agents have improved response rates of women with ovarian cancer.(2-7) Despite these advances, no significant improvement in overall survival has been demonstrated.(1) Patients with advanced disease treated with a platinum/taxane-based chemotherapy will experience partial response rates over 80% and complete response rates as high as 60%.(8) Unfortunately, recurrence occurs in about 70% of patients who have a complete clinical response, and 90% of patients who recur will develop resistance to platinum-based chemotherapies.(5, 9)

More than 33% of women diagnosed with ovarian cancer have ascites present on initial presentation.(10) Presence of ascites is associated with a poorer prognosis. The ascites cells are convenient for clinical research but also valuable scientifically because they play a role in metastasis at the time of primary occurrence and disease recurrence and in chemoresistance.(10)

Because of the high recurrence rates and resistance to salvage chemotherapy, researchers have investigated novel targeted therapies to overcome platinum resistance. The Wnt/β-catenin pathway is a signaling pathway that mediates ovarian cancer initiation and progression through genes that regulate cell proliferation and apoptosis.(11-13) Preclinical studies have shown an upregulation of Wnt ligands in ovarian cancer ascites, which suggests an increase in Wnt/β-catenin pathway activity.(9) The Wnt/β-catenin

pathway has also been shown to contribute to chemoresistance in ovarian cancer as it promotes epithelial-to-mesenchymal transition.(14)

Porcupine (PORCN) is an enzyme that is crucial to Wnt ligand transport, secretion, and activity.(15) WNT974 (Novartis, Cambridge, MA) is a selective, orally bioavailable PORCN inhibitor that decreases the secretion of Wnt ligands outside the cell leading to a decrease in Wnt ligand cell surface receptor phosphorylation and a decrease in the expression of Wnt target genes.(16) WNT974 has been shown to suppress growth of Wnt-driven tumors, such as pancreatic and head and neck squamous cell cancer with mutations in Wnt pathway modulators such as RNF43.(17) The R-spondin (RSPO) protein family is comprised of four secreted growth factors. Secreted R-spondin (RSPO) proteins activate Wnt/β-catenin signaling, and RSPO mutations can further upregulate Wnt/β-catenin signaling.(18) Cardona et al. found the prevalence of RSPO fusion gene mutations, primarily with RSPO2 and RSPO3, to be as high as 45% in patients with ovarian cancer.(19) Our primary objective was to evaluate the effect of the Wnt/β-catenin pathway inhibitor, WNT974, in primary ovarian cancer ascites cells and evaluate the importance of R-spondins in sensitivity to WNT974.

MATERIALS AND METHODS

Specimen collection and processing

With IRB approval, ascites was collected from 60 ovarian cancer patients. Forty of those patients also had matched tumor specimens collected. Cells were isolated from ascites samples as previously described.(9) Cells were stored in 10% dimethyl sulfoxide (DMSO) and kept frozen in liquid nitrogen for future analysis. A pathologist confirmed

that all samples were papillary serous ovarian or primary peritoneal cancer. The viability of the ascites cells was confirmed with trypan blue staining showing ≥ 74% viability after the cells had been frozen and thawed twice. All experiments were done using cells that had been thawed only once or twice.

Drugs and Antibodies

WNT974 was obtained from Novartis under a material transfer agreement. A stock solution of 10 mM WNT974 was diluted in DMSO at a 5 mM concentration and stored at 4°C. A 25 mM stock solution of carboplatin was prepared in sterile H2O.

ATPlite luminescence-based Assay

Patient-derived ascites cells were plated in a 96-well ultra-low attachment plate (Corning Costar, Corning, NY) at 2,000 cells/well in 45 μL X-vivo serum free media containing 20 ng/mL epidermal growth factor and 10 mg/mL bovine insulin. They were then treated with 2.5 μL of 1 μM WNT974, 2.5 μL carboplatin 100 μM, or combination WNT974 and carboplatin totaling 5 μL. The plates were incubated at 37°C in a humidified 5% CO2 atmosphere and treated with WNT974 on day 2 and carboplatin on day 6 totaling 48 hours of carboplatin treatment. All patient samples underwent testing with 16 replicates, duplicate assays with eight replicates each. On day 8, the cells were lysed and viability was analyzed using the ATPlite luminescence-based assay (PerkinElmer, Waltham, MA) as previously described (9) The ascites samples that showed a decrease in ATP concentration after treatment demonstrated no difference from the untreated cells in percent viability with trypan blue staining. Flow cytometry was performed on treated and untreated ovarian cancer ascites cells. Samples showing ≥ 30%,

10-30%, and <10% reduction in ATP counts compared to the untreated control were deemed WNT974 sensitive, intermediate sensitive, and resistant, respectively.

IC50 and Dose-response Analysis

Ascites cells from the WNT974 sensitive patient samples were prepared as above in 96-well ultra-low attachment plates then treated with WNT974 at varying concentrations (0.25, 0.5, 0.75, 1, 1.5, 1.75, 2, 2.25, 2.5, 3, and 3.5 μM). After 7 days of treatment with WNT974, viability was analyzed using the same ATP-lite luminescence-based assay.

Immunohistochemistry

To compare treated and untreated patient samples, ascites cells were plated in 6-well ultra-low attachment plates (Corning Costar) at 150,000 cells/well then treated with 1 μM WNT974 for 48 hours. Untreated and treated ascites cells were prepared for IHC staining as previously described.(9) Formalin-fixed, paraffin-embedded (FFPE) slides from the untreated tumor and an omental metastatic site collected at the time of the initial surgery were used for IHC staining of the tumor samples. Papillary serous histology was confirmed by hematoxylin and eosin staining. Additional slides from the paraffin block were sectioned at 4 μm for IHC. Slides were melted in a 60°C oven for one hour and the sections were cleared in a graded xylene/ethanol series then rinsed in dH20. The slides underwent antigen retrieval by placing in pre-heated 0.01 M citric acid pH 6 (LRP6) or 10 mM Tris/1 mM EDTA pH 9 (other antibodies) and pressure cooking for 10 minutes at 15 psi. Pressure was allowed to equilibrate and slides were cooled for 10 minutes. Sections were treated with 3% hydrogen peroxide/H2O for 5 minutes to suppress endogenous peroxidase and blocked with 3% goat serum to reduce non-specific binding.

Primary antibodies were diluted in Antibody Diluent Buffer (1% BSA, 1 mM EDTA in PBS) as follows: polyclonal rabbit anti-phosphorylated LRP6 (Bioss Antibodies, Woburn, MA), 1:50, polyclonal rabbit anti-PORCN (Novus Biologicals, Littleton CO), 1:50, monoclonal rabbit anti-E-cadherin (ThermoFisher, Waltham, MA), 1:100, monoclonal rabbit anti-survivin, clone EPR2675 (Abcam, Cambridge, MA), 1:100, monoclonal mouse anti-β-catenin, clone E-5 (Santa Cruz Biotechnology, Dallas, TX), 1:750. Primary antibodies were incubated for one hour at room temperature. Control multi-tissue slides were used as a positive control for each staining run. The secondary detection system was EnVision + System HRP Labeled Polymer Anti-Rabbit or Anti-Mouse (Dako, Carpinteria, CA). DAB substrate (Scytek Laboratories, Logan, UT) was used to visualize antibody-antigen interaction and then counterstained with Mayer's Hematoxylin Solution, dehydrated through alcohols and xylenes, and mounted using permount. Once IHC staining was completed, the intensity of the staining for β-catenin, E-cadherin, survivin, PORCN, and phosphorylated-LRP6 was graded on a scale of 0 (no staining) to 4 (intense staining). The weighted average of the intensity score was calculated by multiplying the percentage of cells at each intensity by the decimal equivalent of that percentage. This weighted average is considered the immunostaining score.(20) The intensity of each ascites and tumor sample was reported by a pathologist who was blinded to treated versus untreated ascites cells.

Taqman Assays

For tumor tissue homogenization, samples were weighed and placed in 15 mL conical tubes containing 1.4 mm ceramic beads (VWR cat. # 10032-374). The tissue samples were suspended in 35 µL of buffer (Qiagen, Valencia, CA) plus 1% BME per

gram of tissue. The samples were then agitated on a MP Biomedicals FastPrep machine for three cycles of 45 seconds at full speed. Homogenized samples were stored at -80°C until use. Ascites cell samples were thawed and an aliquot was counted. Cell counts ranged from 30,000 to 96 x 106 per sample. Cells were then centrifuged at 200xg for two minutes, and the supernatant removed. A minimum of 700 µL of buffer plus 1% BME was added to each sample. When necessary, additional buffer was added to dilute the sample to approximately 8 x 106 cells/mL. Cell lysates were stored at -80°C until use.

RNA was extracted from 350 µL of tumor or ascites cell lysate using the Norgen Total RNA Purification Kit (Norgen Biotek, Ontario, Canada). Tumor samples were treated with Proteinase K prior to extraction, and the on-column DNase digest was performed according to the manufacturer's instructions. After elution, total RNA was quantified using a Qubit 2.0 fluorometer and RNA HS Assay Kit (Invitrogen, Carlsbad, CA). Reverse transcription was performed on 500 ng of total RNA or 10 µL of extracted total RNA in the case of low-yield samples using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Green Island, NY). The reaction was performed in a Bio Rad T100 thermal cycler programmed according to kit instructions. DNA was extracted from 350 µL of tumor or ascites cell lysate using the Qiagen DNeasy Blood & Tissue Kit. RNase digestion was omitted, but extraction was otherwise performed according to manufacturer's instructions. After elution, DNA was quantified using a Qubit 2.0 fluorometer and dsDNA HS Assay Kit (Invitrogen).

Pre-mixed TaqMan assays were ordered from Applied Biosystems for control genes (ACTB, GAPDH, and FCGRT), PORCN, RSPO2, RSPO3, AXIN2 and Wntless (WLS). Forward and reverse primers and TaqMan probes were designed for the 3 gene

fusions E1F3E (e1)-RSPO2(e2), PTPRK(e1)-RSPO3(e2), and PTPRK(e7)-RSPO3(e2). Positive controls were included via genomic DNA, synthetic oligos (IDT) or cDNA clone (Transomic, Huntsville, AL). For low-yield samples, a 1:5 dilution of cDNA was used. Otherwise, a 1:10 dilution was analyzed. qPCR for all eleven genes: 3 gene fusions, 3 control genes, and RSPO2, RSPO3, AXIN2, WLS and PORCN expression was performed in triplicate on each cDNA sample. Each 10 µL reaction was prepared using TaqMan Universal Master Mix (Applied Biosystems). Samples were run in a 384-well plate on an ABI Prism 7900HT Sequence Detection System running SDS 2.4 software. Control values were computed automatically. Relative quantification of transcripts were calculated first by normalizing to housekeeping genes and then by normalizing for amplicon-specific amplification based on standard curves.

Statistical Analysis

The ratio of ATP levels (counts per second) for treated versus untreated control ascites cells (percent control) was calculated. Statistical analysis comparing ATP levels was performed using SAS version 9.2. A mean percent viability was calculated from all replicates of the control group receiving no treatment. This value was then divided by itself to give a null value of one. The proportion of viability for each treatment group was then compared to the null value of one using a one-tailed t-test to show the difference in viability. Significance was determined when the p-value was < 0.05. The WNT974 IC50 (half maximum inhibitory concentration) was defined as the log10 concentration generating a 50% reduction in ATP levels when compared to the untreated control. All ATPlite analysis represents 16 replicates and IC50 data represents eight replicates. Mean ± standard error is represented in the error bars as detailed in the figure legends. Fisher's

exact, Chi-square, and student's t-test were used to determine the statistical significance of descriptive statistics. A comparison of RSPO2, RSPO3, and PORCN gene expression to ascites sample sensitivity was performed using a two-way ANOVA test. Paired t-test was used to calculate changes in AXIN2 levels following treatment with WNT974 compared to matched untreated samples. Significance was assigned when the p-value was < 0.05.

RESULTS

Ascites samples from 60 patients were analyzed. Patient demographics are outlined in Table 1. The mean age was 64 years old, and the majority of patients were white (85%). Fifty (83.3%) patients were diagnosed with ovarian cancer and 10 (16.7%) with primary peritoneal cancer. All but one patient had primary disease. RECIST 1.1 was used to determine response rates after chemotherapy. Thirty-two (53.3%) patients had a complete response and 13 (21.7%) patients had a partial response. Response rates were unable to be determined in 11 (18.3%) patients due to incomplete medical records, death before completing chemotherapy, or the patient is still receiving adjuvant chemotherapy. In those 32 women who experienced a complete response, 10 (31.3%) recurred with a median time to recurrence of 9.5 months.

Cell viability analysis was performed on all 60 patient ascites samples. Seven patient samples were excluded because there were not enough tumor cells in the thawed samples to proceed with testing. Figure 1 shows the range, 0 to 97%, of the decrease in ATP concentration expressed as a percent of untreated control for 53 samples when treated with 1 μM WNT974. The results of the ATPlite analysis are grouped into three different response groups in Figure 2. Few samples responded to WNT974 alone, but a

large fraction of patient samples showed significant loss of ATP when WNT974 was combined with carboplatin. This ATP loss was greater than treatment with carboplatin alone. Table 2 shows the relationship between the response groups in vitro to their actual clinical response. Despite the limited response to single agent WNT974, 32/51 (63%) of patient samples showed ≥ 30% reduction in ATP concentration with combination treatment. Patients with both platinum sensitive and platinum resistant disease showed strong response to combination treatment with 73% and 50%, respectively, showing high levels of ATP reduction. There was no difference in the sensitivity to WNT974 alone between the platinum sensitive and platinum resistant patient samples. Only 2 of the 14 (14%) samples of cells isolated from platinum resistant patient samples treated with carboplatin alone had a ≥ 30% reduction in ATP concentration; whereas, 12 of the 26 (46%) of the cells isolated from platinum sensitive patient samples had a ≥ 30% reduction in ATP concentration suggesting that there is a correlation between clinical outcomes and in vitro results, although they do not correspond perfectly.

IC50 and dose response analysis was performed on sensitive patient ascites samples (patient samples that had a ≥ 30% reduction in ATP concentration). Representative samples are shown in Figure 3 for patients OV-7 and OV-41 treated with a range of doses. The IC50 for OV-7 was 1.14 μM and 1.76 μM for OV-41. When correlating these samples to the results in Figure 1 where 1 μM WNT974 was used in the ATPlite analysis, OV-7 shows a 50% reduction in ATP counts with 1 μM WNT974 while there was only a 33% reduction in ATP count for OV-41 when using 1 μM WNT974. A 50% reduction could be obtained after increasing the dose of WNT974 to 1.75 μM as shown in Figure 3.

The ascites samples that showed a decrease in ATP concentration after treatment demonstrated no difference from the untreated cells in percent viability with trypan blue staining, suggesting the WNT974 was not cytotoxic. Flow cytometry demonstrated fewer cells in the G2 phase and more in the G1 and S phases after 2 or 7 days of treatment with WNT974, supporting cell cycle arrest as the mechanism of action of WNT974 (Figure 4).

IHC analysis of β-catenin, E-cadherin, PORCN, survivin, and phosphorlyated-LRP6 was performed on the primary tumor, a metastatic tumor implant, and both untreated ascites cells and those cells treated with WNT974 only from 6 different patient samples. β-catenin membrane expression is shown in Figure 5A. E-cadherin membrane staining shows no difference between the primary tumor and the metastatic site or between the untreated and the treated ascites (Figure 5B). There were similar levels of cytoplasmic PORCN expression in both tumor and ascites obtained from the same patient (Figure 5C). This was also true for both cytoplasmic and nuclear staining of Survivin, although the nuclear staining was stronger than the cytoplasmic staining (Figure 6A-B). There was a decrease in p-LRP6 in the treated ascites samples compared to the untreated samples, but this was not consistent throughout all 6 samples (Figure 6C-D). Treatment with WNT974 did not change the expression of β-catenin, E-cadherin, PORCN, survivin, or phosphorlyated-LRP6 measured by IHC. We did, however, observe that in twenty ascites samples WNT974 treatment decreased the levels of AXIN2 transcript compared to matched untreated ascites samples, confirming that WNT974 does reduce signaling through the Wnt/β-catenin pathway ($p=0.0002$; Figure 7).

We next asked whether the variability in WNT974 sensitivity could be explained by changes in gene expression in the ascites samples. We measured expression of

WNT974's target, PORCN and key Wnt pathway genes RSPO2 and RSPO3 and three previously characterized fusions involving RSPO2 and RSPO3, which are thought to increase levels of those transcripts. Using custom Taqman assays we measured expression of these genes and fusions and assessed whether expression correlated with sensitivity to WNT974 (Figure 8). Analysis was done on 40 matched ascites and tumor samples and an additional 13 ascites samples without matching tumor tissue. We found that RSPO fusions were detectable in only 1 sample, indicating that they do not play a role in these patient samples. In both ascites and tumor cells an increase in RSPO3 expression was associated with sensitivity to the combination of WNT974 and carboplatin, but not carboplatin or WNT974 alone (Figure 8A). Similarly, increased WLS expression in ascites correlated with sensitivity to WNT974 alone ($p=0.0399$), trended with sensitivity to carboplatin alone and to the combination treatment ($p=0.065$), but WLS expression in tumors did not correlate with sensitivity (Figure 9). This suggests an upregulation of the Wnt/β-catenin pathway in platinum-resistant ovarian cancer, particularly in metastatic cells. This supports previous studies showing upregulation of the Wnt/β-catenin pathway in platinum-resistant ovarian cancer.(9, 14) In addition, PORCN gene expression correlates with ascites cell sensitivity to WNT974 (Figure 8B). Higher PORCN expression was measured in the untreated ascites samples that produced a $\geq 30\%$ reduction in ATP when treated with WNT974 compared to those ascites samples that did not show a drop in ATP after WNT974 treatment ($p=0.0073$). This suggests that cells with higher endogenous levels of PORCN and WLS are more sensitive to WNT974.

DISCUSSION

The Wnt/β-catenin pathway has been shown to contribute to ovarian cancer initiation, metastasis, chemoresistance, and recurrence.(9, 11, 12) Evidence supports the presence of cancer stem cells (CSCs) in the development of tumor chemoresistance and recurrence, and upregulation of the Wnt/β-catenin pathway found in CSCs is thought to contribute to CSC maintenance.(9, 21) Increased activity of the Wnt/β-catenin pathway can result from upregulation at different steps in the signaling pathway: overexpression of the cell surface receptors LRP5, LRP6, and Frizzled or increased activity of the Wnt target genes survivin, cyclin D1, Axin2, and c-myc.(9, 13, 22) A crucial component necessary for Wnt ligand transport, secretion, and activity is PORCN, which has been identified as a potential target to inhibit Wnt/β-catenin signaling.(15)

Wnt pathway inhibitor success has been limited because of a narrow therapeutic window for activity since normal tissue stability requires Wnt/β-catenin signaling and a lack of predictive biomarkers of response. WNT974 inhibits Wnt/β-catenin signaling through inhibition of the PORCN enzyme which causes a decrease in secretion of Wnt ligands. The prevention of Wnt ligand secretion after treatment with WNT974 results in decreased phosphorylation of the surface receptor LRP6 and decreased expression of Wnt target genes like AXIN2.(16, 17) WNT974 has shown anti-tumor activity in pancreatic adenocarcinoma and head and neck squamous cell cancer. Jiang et al. identified both WNT974 sensitive tumor cells and a potential predictive biomarker after demonstrating tumor growth inhibition through cell cycle arrest in in vivo pancreatic tumors treated with WNT974 with inactivating mutations in the Ring finger 43 (RNF43) gene.(17) Wnt/β-catenin signaling inhibition was shown through decreased expression of the target genes

Axin2 and c-myc. Liu et al. found a dose-dependent tumor growth delay in human head and neck squamous cell cancer xenografts treated with WNT974. (16) Evidence of Wnt/β-catenin signaling inhibition was shown with decreased Axin2 expression and prevention of LRP6 receptor phosphorylation. They also discovered a correlation between a loss of function Notch1 gene mutation and WNT974 sensitivity, which may also indicate a potential predictive biomarker for WNT974.

The presence of ascites is a predictor of poor outcomes in women diagnosed with ovarian cancer.(10) Certain populations of tumor cells in ascites show CSC-like properties, chemoresistance, and the ability to promote distant metastasis and recurrence.(10) The ascites fluid creates a microenvironment containing the necessary components to promote tumor cell growth.(10) Since ascites is often present at the time of diagnosis and recurrence, this fluid provides an ideal opportunity to evaluate these tumor cells and advance our understanding of ovarian cancer pathogenesis.

We evaluated the effect of WNT974 on primary ovarian cancer ascites cells isolated from 53 patient samples with papillary serous ovarian cancer. Trypan blue staining showed no change in the percent viability after treatment with WNT974 suggesting that it did not cause cytotoxicity to the cells. Flow cytometry results support cell cycle arrest as the mechanism of action for WNT974 in ovarian cancer ascites cells. IHC analysis of E-cadherin showed similar levels of expression in both the untreated and treated ascites cells suggesting a lack of epithelial-to-mesenchymal transition. Reduction in AXIN2 expression indicates inhibition of the Wnt/ β-catenin pathway. Cell cycle arrest was demonstrated on ATPlite analysis with a $\geq$ 30% reduction in ATP concentration in 13% of patient samples treated with WNT974 only and 30% of patient

samples treated with carboplatin only. The number of samples that showed a ≥ 30% reduction in ATP concentration increased to 60% when WNT974 was combined with carboplatin treatment. Similar IHC staining of the Wnt pathway proteins β-catenin, survivin, and phosphorylated-LRP6 and the PORCN enzyme between tumor and both untreated and treated ascites cells suggests cytostasis rather than cytotoxicity with WNT974 treatment.

Despite a previous preliminary report of RSPO fusion transcripts in ovarian cancer, we found little evidence for RSPO fusions in either tumor or ascites samples.(19) Our efforts to reveal gene expression patterns that correlate with sensitivity to WNT974 showed that there was also no correlation between RSPO3 gene expression and sensitivity to WNT974 alone but increased RSPO3 levels were associated with response to the combination of WNT974 and carboplatin, suggesting that WNT974 makes chemotherapy resistant cells more sensitive to carboplatin. We did demonstrate increased RSPO3 gene expression in samples resistant to carboplatin in support of previous studies demonstrating upregulation of the Wnt/β-catenin pathway in platinum-resistant cancer cells.(9, 14) PORCN expression may be a potential indicator for patients who would respond to WNT974 because higher PORCN expression was seen in the patient ascites samples with ≥ 30% reduction in ATP on ATPlite analysis.

The WLS gene is a key modulator of Wnt protein expression that is overexpressed in some ovarian cancers.(23) We observed that WLS expression was lower in ascites compared to tumor but that increased WLS expression in ascites correlated with sensitivity to the WNT974 + carboplatin combination.

There are several strengths of this study including the large number of samples tested, specifically the 40 matched tumor and ascites specimens. All but one of the samples collected in this study were obtained at the time of primary diagnosis. Comparing both ascites and tumor gene expression allowed us to determine that biomarkers of drug sensitivity were similar in ascites and tumor. Using ascites cells in this study allowed us to study their microenvironment which may create chemoresistance.(24) In the future, we plan to evaluate samples from patients with recurrent disease since Wnt/β-catenin signaling may be further upregulated in patients with recurrent disease.(9, 14)

Wnt inhibitors are now being evaluated in several clinical trials. A Phase I trial using LGK974, renamed WNT974 by Novartis, opened in 2011 for patients with pancreatic adenocarcinoma, BRAF mutant colorectal cancer (CRC), and other tumors with documented genetic mutations upstream in the Wnt/β-catenin signaling pathway (NCT01351103). Patients with BRAF mutated metastatic CRC and other Wnt pathway mutations are eligible for a Phase Ib/II trial evaluating the safety and activity of the combination of WNT974, the RAF kinase inhibitor LGX818, and cetuximab (NCT02278133). This clinical trial evaluates multiple targeted therapies which may provide better clinical outcomes than a single agent. Phase II trials combining these targeted agents with standard chemotherapy may be warranted given our finding of improved response with a combination of WNT974 and carboplatin.

CONCLUSION

The Wnt/β-catenin pathway is an important signaling pathway that lends itself to pharmacologic intervention through mechanisms different than cytotoxic chemotherapy. Through modification of proteins on the cell surface, in the cytoplasm, and in the nucleus, we have shown activity of the Wnt/β-catenin pathway inhibitor WNT974 in patients with papillary serous ovarian cancer. Genetic analysis of these ascites samples has identified a potential predictive biomarker for patients that may respond to WNT974 in elevated PORCN expression. The toxicity of WNT974 in patients with peritoneal disease is under investigation. Ultimately, the combination of targeted agents like WNT974 with cytotoxic chemotherapy may prove to be an effective option for patients with recurrent ovarian cancer.

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Table 1 Patient demographics

	N = 60 (%)
Race	
White	51 (85)
African American	8 (13.3)
Hispanic	1 (1.7)
Mean age (± s.d.)	64 (10.1)
Mean BMI (± s.d.)	28.9 (6)
Site of malignancy	
Ovary	50 (83.3)
Primary peritoneal	10 (16.7)
Histology	
Papillary serous	60 (100)
Disease status	
Primary	59 (98.3)
Recurrent	1 (1.7)
Clinical response to treatment	
Complete response	32 (53.3)
Partial response	13 (21.7)
Progression	4 (6.7)
Unknown	11 (18.3)
Recurred after complete response	10 (31.3)
Time to recurrence	
Median (range)	9.5 (2–15)
Clinical status at last follow-up	
No evidence of disease	27 (45)
Alive with disease	11 (18.3)
Deceased	12 (20)
Unknown	10 (16.7)

Table 2 Patient ascites sensitivity after drug treatment compared with the clinically observed response

Decrease in ATP (% Control)	Platinum sensitive	Platinum resistant	Unknown	
	$N=26$	$N=14$	$N=13$	
WNT974				$P=0.6723$
≥30	2	3	2	
10–30	10	4	6	
<10	14	7	5	
Carboplatin				$P=0.1557$
≥30	12	2	2	
10–30	10	10	8	
<10	4	2	3	
WNT974				$P=0.3446$
+carboplatin				
≥30	19	7	6	
10–30	6	5	5	
<10	1	2	2	

Ascites cells from ovarian cancer patients treated with 1 μM WNT974 for 7 days, 100 μM carboplatin for 48 h, or combination WNT974 and carboplatin are stratified by their clinical response to platinum-based chemotherapy. Patients were categorized as platinum sensitive or resistant. More patient samples demonstrated a ≥30% reduction in ATP concentration and fewer samples showed a <10% reduction in ATP concentration with combination therapy compared with treatment with either single agent alone.

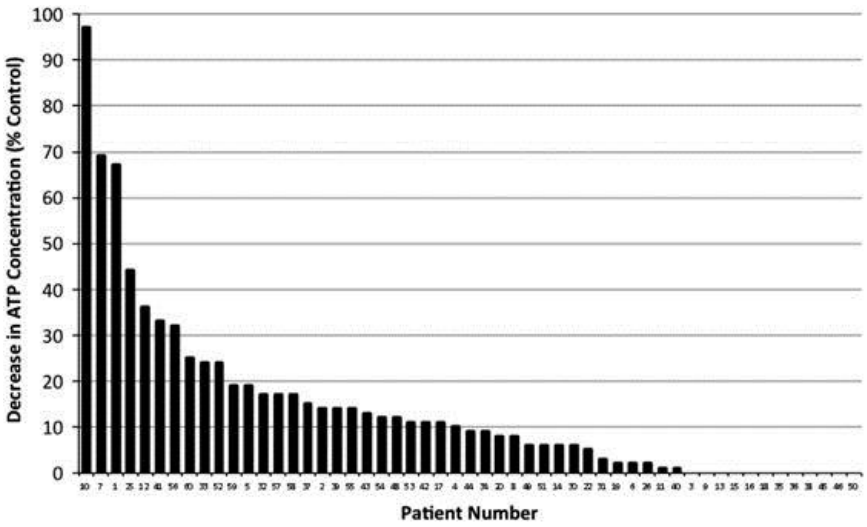

Figure 1. Patient sample sensitivity. Fifty-three ovarian cancer ascites samples were treated with 1 μM WNT974 for 7 days. Sensitivity was analyzed using the ATPlite luminescence-based assay.

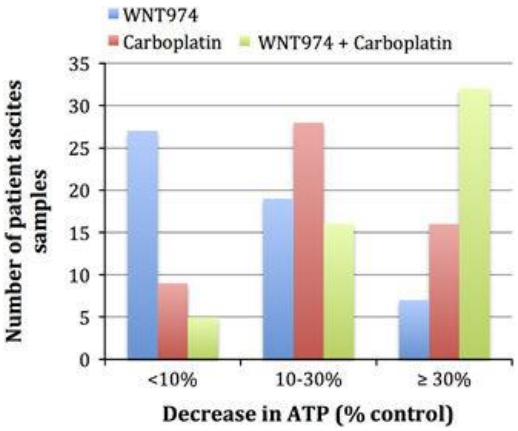

Figure 2. Sensitivity of patient ascites samples to WNT974. Fifty-three ovarian cancer ascites samples were treated with 1 μM WNT974, 100 μM carboplatin, or a combination of WNT974 and carboplatin. Viability was analyzed using the ATPlite luminescence-based assay. The number of tested samples showing each range of decrease in ATP is shown.

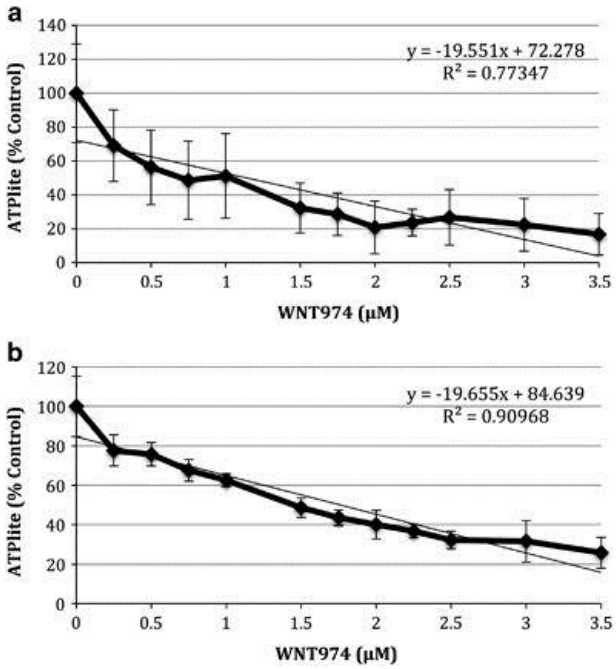

Figure 3. WNT974 IC50 dose–response curves. Tumor cells isolated from the ascites of patients OV-7 (a) and OV-41 (b) were treated with WNT974 (0.25 to 3.5 μM) for 7 days. Sensitivity was measured with ATPlite. The IC50 value was determined. Error bars represent s.e.m. for eight replicates.

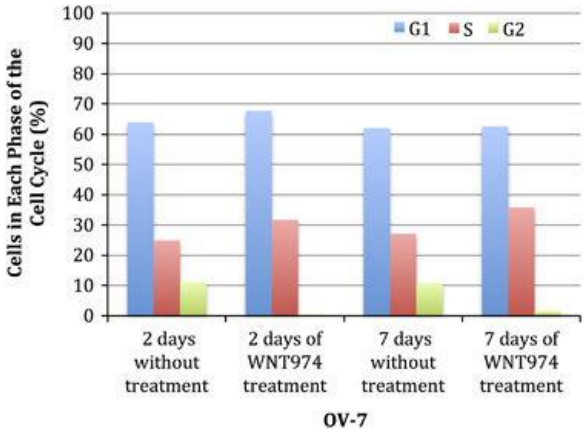

Figure 4. Cell cycle analysis of patient ascites samples. Flow cytometry was performed on patient ascites samples treated with 1 μM WNT974 and those left untreated after 2 days and 7 days. A representation of the results is shown using ascites cells from patient OV-7. Treatment caused a decrease in the number of cells in the G2 phase and an increase in the number of cells in the G1 and S phases after both 2 and 7 days of treatment.

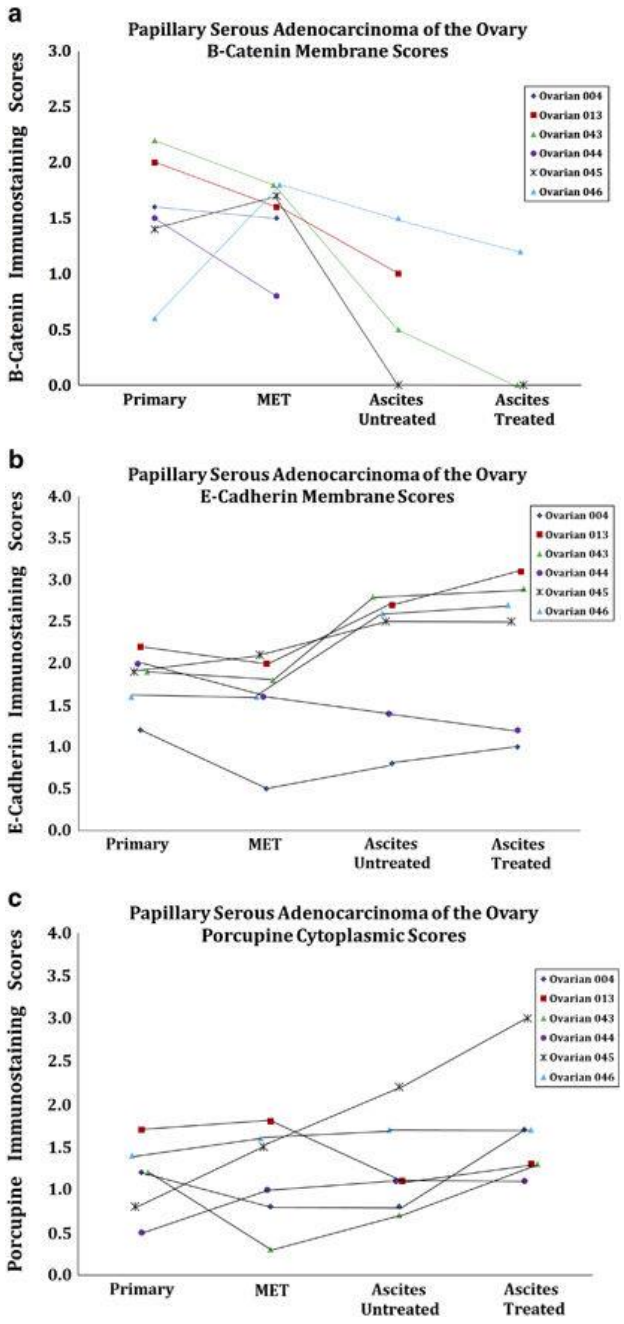

Figure 5. Immunohistochemical analysis of β-catenin, E-cadherin, and PORCN. IHC analysis of β-catenin (a), E-cadherin (b), and PORCN (c) was performed on the primary tumor, a metastatic tumor implant (MET), and both untreated and WNT974-only treated ascites tumor cells from six different patient samples. The intensity of staining with β-catenin and E-cadherin (a and b) showed similar levels of membranous protein expression among both tumor and ascites cells obtained from the same patients. There were similar levels of PORCN cytoplasmic protein (c) expression among both tumor and ascites cells obtained from the same patient. IHC, immunohistochemistry.

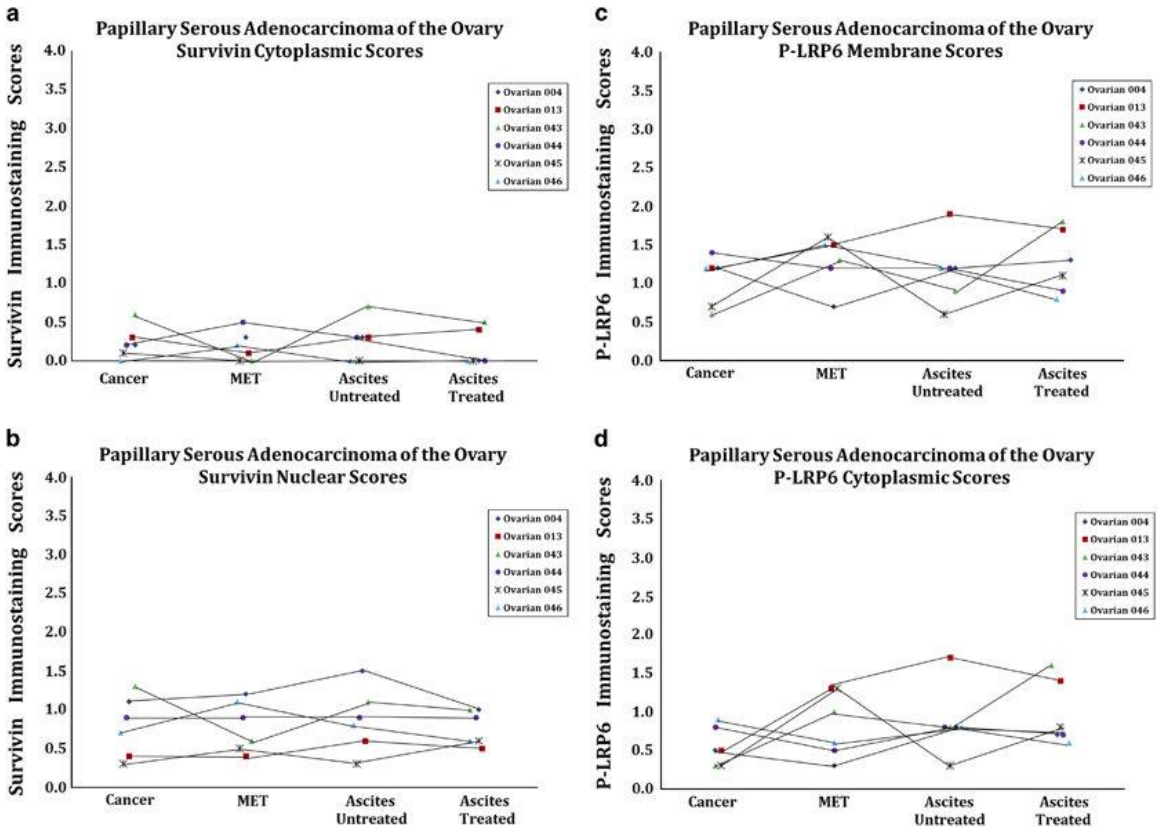

Figure 6. Immunohistochemical analysis of survivin and p-LRP6. IHC analysis of survivin and p-LRP6 was performed on the primary tumor, a metastatic tumor implant (MET), and both untreated and WNT974-only treated ascites tumor cells from six different patient samples. There was similar levels of cytoplasmic (a) and nuclear (b) survivin protein expression among both tumor and ascites cells obtained from the same patients. There were similar levels of p-LRP6 membranous (c) and cytoplasmic (d) protein expression among both tumor and ascites cells obtained from the same patients. IHC, immunohistochemistry.

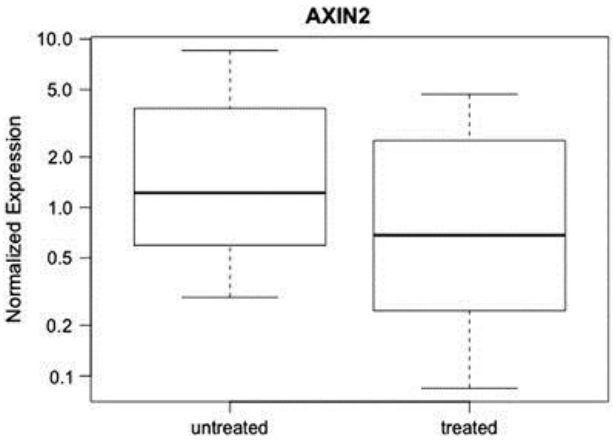

Figure 7. AXIN2 transcript levels in response to WNT974 treatment. The level of AXIN2 transcript was measured in 20 samples of both untreated and matched ascites treated with WNT974. There was a decreased level of AXIN2 transcript in treated ascites confirming that WNT974 reduces signaling through the Wnt/β-catenin pathway. Paired t-test was used to calculate changes in AXIN2 levels following treatment with WNT974 compared with matched untreated samples.

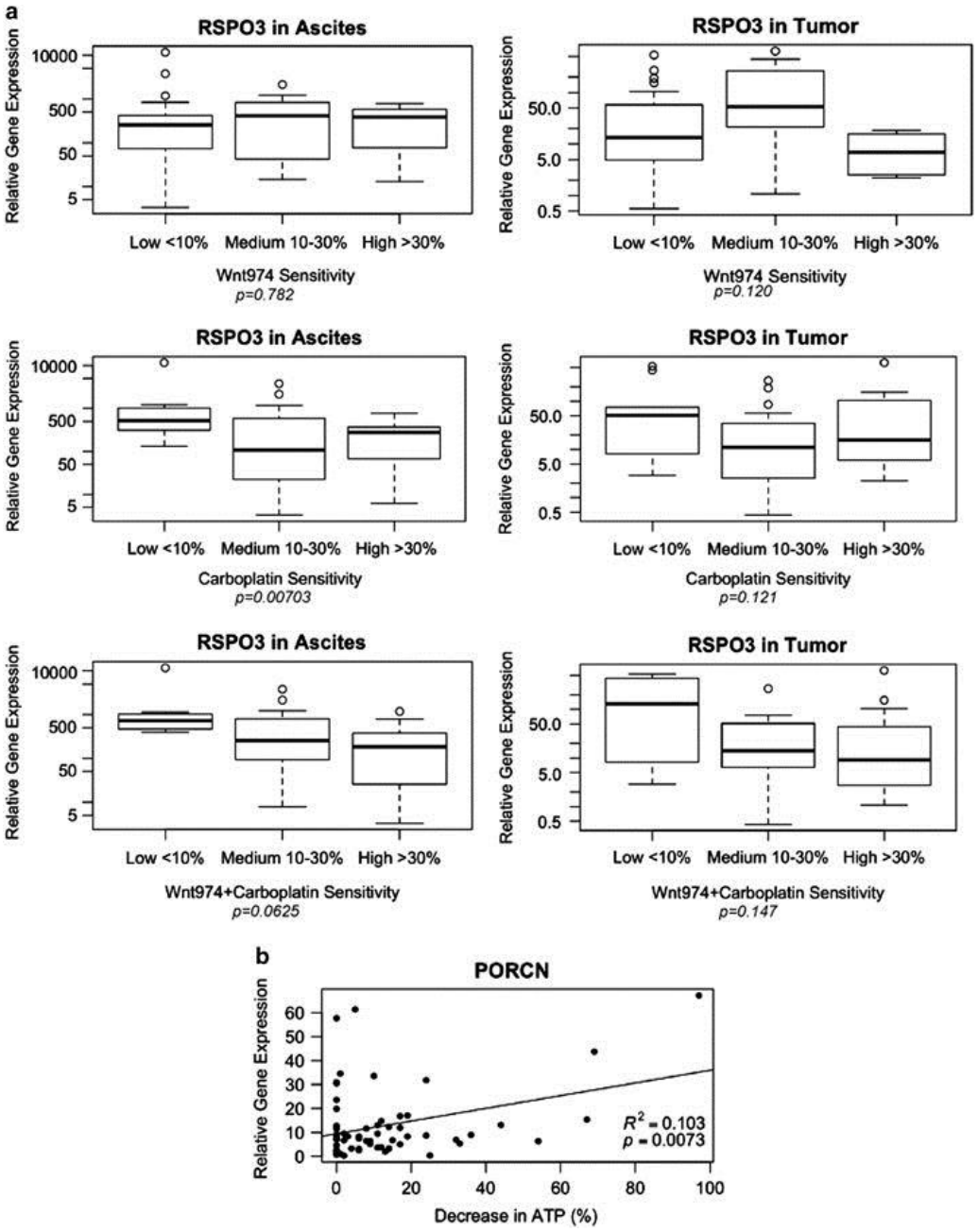

Figure 8. Gene expression in ovarian cancer patient samples. (a) RSPO3 expression before treatment with carboplatin and WNT974 or carboplatin only is compared with the response in ascites cells after treatment. High sensitivity indicates a ≥ 30% reduction in ATP concentration, medium sensitivity indicates a 10–30% reduction, and low sensitivity indicates a o10% reduction. Samples with the lowest response to WNT974 and carboplatin had higher RSPO3 expression at baseline. (b) PORCN gene expression in untreated ascites cells is compared with ascites sensitivity to WNT974. Higher PORCN expression was correlated with greater decrease in ATP concentration. A Pearson's correlation between PORCN and viability after WNT974 treatment was calculated, and the P-value calculated using default R parameters.

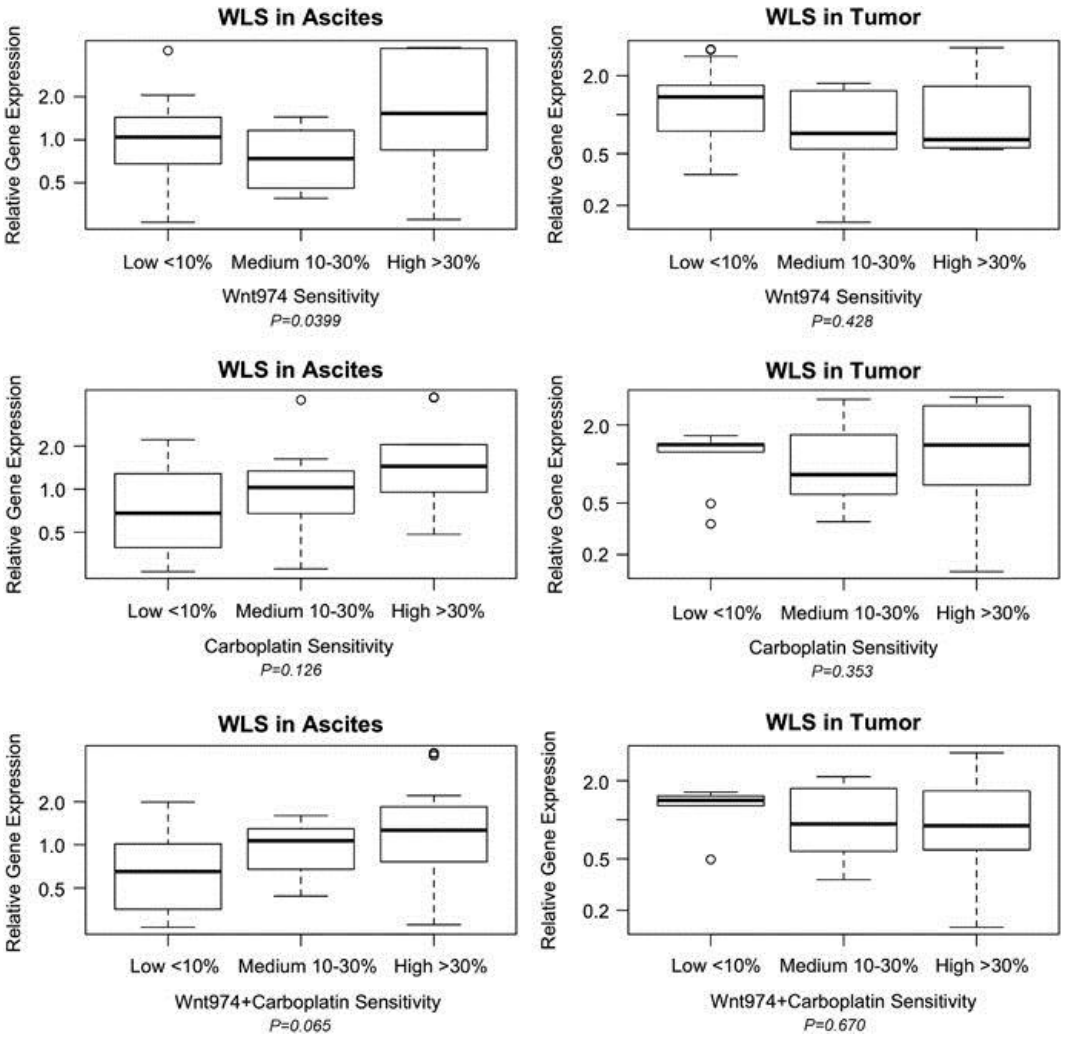

Figure 9. WLS expression in ovarian cancer ascites and tumor. Increased WLS expression was seen in ascites samples sensitive to WNT974 alone. WLS expression only trends with sensitivity to carboplatin alone and to combination WNT974+carboplatin treatment. In tumor samples, WLS expression did not correlate with sensitivity.

NICLOSAMIDE AND ITS ANALOGS ARE POTENT INHIBITORS OF WNT/β-CATENIN, mTOR, AND STAT3 SIGNALING IN OVARIAN CANCER

by

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Oncotarget

Errata corrected and format adapted for dissertation

ABSTRACT

Epithelial ovarian cancer (EOC) is the leading cause of gynecologic cancer mortality worldwide. Platinum-based therapy is the standard first line treatment and while most patients initially respond, resistance to chemotherapy usually arises. Major signaling pathways frequently upregulated in chemoresistant cells and important in the maintenance of cancer stem cells (CSCs) include Wnt/β-catenin, mTOR, and STAT3. The major objective of our study was to investigate the treatment of ovarian cancer with targeted agents that inhibit these three pathways. Here we demonstrate that niclosamide, a salicylamide derivative, and two synthetically manufactured niclosamide analogs (analog 11 and 32) caused significant inhibition of proliferation of two chemoresistant ovarian cancer cell lines (A2780cp20 and SKOV3Trip2), tumorspheres isolated from the ascites of EOC patients, and cells from a chemoresistant patient-derived xenograft (PDX). This work shows that all three agents significantly decreased the expression of proteins in the Wnt/β-catenin, mTOR and STAT3 pathways and preferentially targeted cells that expressed the ovarian CSC surface protein CD133. It also illustrates the potential of drug repurposing for chemoresistant EOC and can serve as a basis for pathway-oriented *in vivo* studies.

INTRODUCTION

Epithelial ovarian cancer (EOC) was responsible for an estimated 14,000 deaths in the United States in 2015 making it the deadliest gynecologic malignancy [1]. Surgical resection in combination with platinum and taxane-based chemotherapy is the standard treatment for patients with EOC. Although the majority of patients with EOC become

clinically disease-free after initial treatment, 75% of patients ultimately recur within 5 years. Developing more effective and durable treatments is a significant unmet medical need in this disease context. A subset of cells may survive first-line chemotherapy and ultimately be responsible for the development of clinically detectable recurrence. These cells may be cancer stem cells (CSCs) that are resistant to primary therapy and are responsible for the generation of progeny cancer cells [2].

Important pathways that are frequently upregulated in CSCs are the Wnt/β-catenin signaling pathway, the mammalian target of rapamycin (mTOR) pathway, and the signal transducer and activator of transcription-3 (STAT3) pathway [3, 4]. All three of these pathways have been shown to be associated with recurrence and development of chemoresistance in ovarian cancer [5, 6]. A well established in vitro model has been used to replicate the behavior of CSCs, which involves plating cells in serum-free media that contains growth factors in low-attachment plates so that they form spheres rather than adhere to the plate as single cells. Studies have shown that these tumorspheres have increased expression of ovarian CSC markers such as CD133 and ALDH1A1 [7]. When these cells are implanted into mice they are more tumorigenic than the bulk population of tumor cells. In order to successfully prevent ovarian cancer recurrence, it is imperative that therapies are developed to specifically target these chemoresistant CSCs. A therapeutic intervention that targets both the Wnt/β-catenin and mTOR/STAT3 pathways provides a promising approach towards the eradication of CSCs.

The traditional drug-development process takes a tremendous amount of time and is extremely costly with a very high failure rate. It has been estimated that it requires approximately a billion dollars and 10 years for a drug to be developed and placed on the

market [8]. Drugs that have previously been used with known pharmacokinetics, pharmacodynamics and toxicity profiles provide an advantage over new drug discovery. New applications can be found for existing drugs, called drug repurposing, which can be valuable especially in diseases such as EOC. Niclosamide (trade name Niclocide) is a salicyclamide derivative in the antihelminth family which has been approved by the U.S. Food and Drug Administration for the treatment of tapeworms. This safe, inexpensive drug has been used in humans for nearly 50 years. Several investigators have independently performed quantitative high-throughput screening of > 4,000 clinically approved compounds and found niclosamide to be a potent anti-cancer compound [9, 10]. Screening of the NCI 60 human tumor cell line panel identified niclosamide as an inhibitor of the Wnt/β-catenin pathway [9]. Niclosamide is a potent mitochondrial uncoupler which can have an effect on cell-cycle arrest and apoptosis [9]. One criticism for pursuing the use of niclosamide as an anti-cancer drug is it poor water solubility (0.23 μg/mL) and poor systemic bioavailability (~10%) [11]. Our group and others have made efforts to develop analogs of niclosamide. Analogs 11 and 32 were both synthesized by Dr. Pui-Kai Li at the Ohio State University in an attempt to improve the bioavailability and solubility and potentially enhance niclosamide's inhibition of proliferation. We previously described the structure and synthesis of these two compounds, which are shown in Supplementary Table S4 [12].

In this study, we investigated the effects of niclosamide, analog 11, and analog 32 on ovarian cancer cells and found that niclosamide and its analogs inhibited the Wnt/β-catenin, mTOR, and STAT3 pathways in chemoresistant ovarian cancer cell lines, in cells derived from a chemoresistant ovarian cancer patient-derived xenograft (PDX model),

and tumorspheres cultured from cells isolated from the ascites of patients with ovarian cancer. Several other groups have also shown that niclosamide and niclosamide derivatives can inhibit mTOR and STAT3 signaling in various tumor types [13–19]. We previously showed that niclosamide and its analogs were anti-proliferative and targeted the Wnt pathway in >30 primary ovarian cancer patient ascites samples, some of which were clinically platinum resistant [12, 20]. In addition, we found that niclosamide specifically decreased the stem cell marker ALHD1A1 and the Wnt pathway surface receptor LRP6. In this current study we demonstrate that niclosamide not only targets the Wnt pathway, but it also targets the mTOR and the STAT3 pathways and specifically targets CD133+ CSCs and chemoresistant cells isolated from a PDX ovarian cancer model.

RESULTS

Anti-proliferation effects of niclosamide and its analogs, as single agents and in combination with chemotherapy

Human ovarian cancer cell lines, A2780ip2 and SKOV3ip1 along with their platinum and taxane resistant derivatives A2780cp20 and SKOV3Trip2 **(**Supplementary Figure 4) were treated with niclosamide, analog 11, or analog 32 (0.1- 4 μM) for 48 h, and cell proliferation was assessed by measuring ATP levels using the ATPlite assay. All three agents produced similar inhibition of proliferation, with IC_{50} values ranging from (0.41- 1.86 μM) (Fig 1A and Supplementary Table 1). These values are well below the known C_{max} of niclosamide in humans of 18.34 μM [21]. Analog 11 had slightly more anti-proliferative activity than the other two compounds for both the SKOV3ip1 parental

cell line and SKOV3Trip2 taxane resistant line, but similar inhibition of proliferation was observed in the A2780ip2 and A2780cp20 cell lines. IC_{50} values for analog 32 were similar to niclosamide in all four cell lines.

We then investigated the effects of combining niclosamide, analog 11 or 32 with chemotherapy. The combination of niclosamide, analog 11, or analog 32 with carboplatin doses ranging from 50 -150 μM produced significantly greater inhibition of proliferation than either agent alone and statistical analysis indicated that the combination of the IC_{50} dose of carboplatin (150 μM) was synergistic with a combination index less than 1 (CI < 1) for all 3 agents in the A2780cp20 cell line (Fig 1B). Synergy was also observed with all 3 agents in the SKOV3ip1 and SKOV3Trip2 cell lines when combined with the IC_{50} dose of carboplatin, 100 μM and 50 μM respectively (Supplementary Fig 1). In the parental A2780ip2 cell line, niclosamide and analog 32 were synergistic with 50 μM of carboplatin, while analog 11 had an additive effect (CI = 1) (Supplementary Fig 1).

The anti-proliferative effects of niclosamide were validated in the two chemoresistant cell lines. Niclosamide inhibited proliferation in a time- and dose-dependent manner. When the difference in cell proliferation between treated and untreated cells was evaluated over a 72 h period in the two chemoresistant cell lines, 1 μM of niclosamide inhibited growth by 24 h and suspension of growth persisted for 72 h (Fig 1C and Supplementary Table 2). Niclosamide at 4 μM showed no increase in cell number after 72 h either due to cell kill, cell cycle arrest, or a combination of both.

In order to investigate if niclosamide and its analogs were targeting the CD133+ population of cells specifically, the chemoresistant A2780cp20 cells, which have a high expression of CD133+ compared to the parental line, were treated for 48 h with 2 μM of

niclosamide, analog 11, or analog 32. At baseline, A2780cp20 cell line contains 80% CD133 + cells compared to 50-60% in the parental A2780ip2 cell line (Supplementary Figure 2B). Analog 32 and niclosamide both caused a statistically significant decrease in the CD133+ population with a 60-80% reduction of CD133+ cells (Fig 1D). There was a slight decrease in the CD133+ population after treatment with analog 11, but this was not statistically significant. Fifty percent of the total cells are killed with an IC_{50} dose of either carboplatin or paclitaxel, but >50% of the surviving cells still express CD133+; in contrast, niclosamide preferentially kills this population (Fig 1D).

Inhibition of the Wnt/β-catenin pathway in ovarian cancer cell lines

In order to evaluate niclosamide's ability to inhibit the Wnt/β-catenin pathway, we measured the nuclear β-catenin driven transcription activation after treatment with 1μM of niclosamide for 24 h using the TOPflash luciferase reporter assay. We observed a statistically significant suppression of β-catenin driven transcription activity in the presence of niclosamide in all four cell lines (Fig 2A). This suppression was also observed in both the parental A2780ip2 and SKOV3ip1 and the chemoresistant A2780cp20 and SKOV3Trip2 cell lines under Wnt stimulation (Fig 2A). Interestingly, we noted that in the chemoresistant lines A2780cp20 and SKOV3Trip2, the addition of Wnt3a failed to show increased activation of β-catenin driven signaling. One explanation for this could be that Wnt/β-catenin is already maximally upregulated in the chemoresistant cells, and the addition of Wnt3a does not further increase the level of nuclear β-catenin. It could also be possible that the Wnt/β-catenin pathway regulation of

these cells is driven by other Wnt ligands and not Wnt3a or that it is deregulated downstream of Wnt stimulation due to other events that stabilize β-catenin.

LRP6 is an essential Wnt co-receptor for the Wnt/β-catenin signaling pathway, and LRP6 phosphorylation is critical for Wnt/β-catenin signaling activation induced by Wnt proteins. Uncomplexed cytosolic β-catenin (free β-catenin) can translocate to the cell nucleus and bind transcription factors leading to the transcription of Wnt target genes. To further evaluate niclosamide and its analogs' effect on the Wnt/β-catenin pathway, we used Western blot analysis to measure the transmembrane receptor LRP6, both total and phosphorylated, and the amount of total β-catenin. Niclosamide and its analogs inhibited endogenous LRP6 expression and phosphorylation in both parental A2780ip2 and platinum resistant A2780cp20 cell lines in a dose dependent manner (Fig 2B). We found that total β-catenin levels in both cell lines were significantly reduced after treatment with niclosamide, analog 11 and analog 32. Although analogs 11 and 32 decreased the expression of phosphorylated LRP6 and total β-catenin at the highest concentration in both cell lines, the decrease in nuclear β-catenin measured by TOPflash reporter assay was only statistically significant in the A2780cp20 cell line, but not in the parental A2780ip2 cell line (Supplementary Fig 2A). This could be the result of the analogs' ability to have an enhanced effect on the more stem-like cells. While analog 11 stimulated total β-catenin levels at 0.5 and 1μM using Western blot analysis in the A2780cp20 cell line, TOPflash reporter assay is a more specific measurement of Wnt/β-catenin pathway activation by measuring the nuclear β-catenin driven transcription activation, which showed a statistically significant decrease with 1μM of analog 11 (Supplementary Fig 2A).

mTOR/STAT3 inhibition of ovarian cancer cell lines and patient samples

Overexpression of downstream mTOR effectors 4E-BP1 and S6K lead to poor cancer prognosis [22]. Activation of mTOR leads to the phosphorylation of P70S6K at threonine 389. In order to evaluate the effect of niclosamide and its analogs on the mTOR pathway, we measured these proteins by Western blot analysis. As demonstrated in Fig 3A, 1 μM of niclosamide inhibited 4E-BP1 and phosphorylated 4E-BP1 in both parental A2780ip2 and chemoresistant A2780cp20 cell lines after 24 h. Analog 11 and analog 32 both inhibited mTOR pathway proteins 4E-BP1, phospho-4E-BP1, p70S6K; however, p(Thr389)-p70-70S6K was only consistently inhibited at the 2 μM dose (Fig 3A).

We have previously published that niclosamide is a potent STAT3 inhibitor in triple negative breast cancer cell lines [23]. To evaluate this finding in ovarian cancer patient samples, we measured total STAT3 and phosphorylated STAT3 after treatment with niclosamide. Niclosamide reduced STAT3 and mTOR downstream protein expression in ascites cells from ovarian cancer patients. STAT3 is phosphorylated by a kinase and translocates to the cell nucleus where it acts as a transcription activator. Specifically, STAT3 becomes activated after phosphorylation of tyrosine 705 in response to specific ligands. Western blot analysis of the phosphorylated (Tyr705) STAT3 protein after 24 h treatment with niclosamide (1 – 8 μM) showed a dose-response inhibition in ovarian cancer patient ascites sample OV-15. In OV-26, OV-05, OV-25 and OV-22, we used the highest dose in an effort to see the most significant effect and the 8 μM concentration of niclosamide completely abolished the phosphorylated (Tyr705) STAT3 protein expression (Fig 3B). Patient sample labeling (i.e. OV- patient #) was kept consistent with our previously published paper that showed dose-response inhibition of

Wnt/β-catenin signaling proteins (LRP6, pLRP6, free and total β-catenin) and 3 target genes (Axin2, survivin, and cyclin D1) [20]. In addition, the mTOR pathway proteins p(Thr389)-P70-70SK and p(Ser235/236)S6 were significantly inhibited in the patient samples (Fig 3B). Overall, niclosamide was more consistent than analog 11 or analog 32 at inhibiting both STAT3 and mTOR protein expression.

Cell cycle arrest and apoptosis by niclosamide

To elucidate the mechanism by which niclosamide inhibited cellular proliferation and caused cell death, we analyzed treated cells for both cell cycle arrest by flow cytometry and ELISA apoptosis assay. All cells were evaluated for their phase in the cell cycle by flow cytometry at 24 and 48 h after treatment with niclosamide. After treating A2780cp20 cells with niclosamide (1- 4 μM), cells arrested at G1 by 24 h at a 1 μM concentration, which was even more evident at higher concentrations (Fig 4A and Supplementary Fig 3).

Apoptosis was determined using an ELISA-apoptosis assay to detect mono- and oligonucleosomes released into the cytoplasm of apoptotic cells as a result of DNA degradation. After treatment with 2 μM of niclosamide, production of apoptotic DNA fragments was significantly increased compared to untreated controls in the A2780ip2, SKOV3ip1 and SKOV3Trip2 cell lines (Fig 4B). Four μM of niclosamide was required to see significant levels of apoptotic DNA fragments in the A2780cp20 chemoresistant cell line. This data suggests niclosamide is causing cell cycle arrest at lower concentrations and causing senescence and cell kill by apoptosis at higher concentrations.

However, necrosis and autophagy, due to mTOR inhibition, could also account for cell kill [15].

Western blot analysis was performed to evaluate the effect of niclosamide and its analogs on the Wnt/β-catenin pathway target genes that affect cell cycle and apoptosis, namely cyclin D1 and survivin. At a concentration as low as 1μM of niclosamide, the proto-oncogene cyclin D1, which controls transition from G1 to S phase, is decreased in both A2780ip2 and A2780cp20 (Fig 4C). Protein levels of survivin, a protein that helps the cell to evade apoptosis, is dramatically reduced in both parental A2780ip2 and chemoresistant A2780cp20 cells at 2 μM (Fig 4C). A potential mechanism for cell cycle arrest and cell kill is the decrease in cyclin D1 and survivin. Similar results were obtained with analogs 11 and 32.

Anti-proliferative effects of niclosamide and analogs 11 and 32 in a PDX model

To confirm our cell line and previously published ascites tumorsphere *in vitro* observations, the effect of niclosamide treatment was evaluated in cells dissociated from an *in vivo* PDX model. A chemoresistant PDX model was created by serial chemotherapy treatments until the xenograft became resistant. Patient 127 had a histology of papillary serous adenocarcinoma and, as previously described in Dobbin *et al*, the histology of the tumor was maintained for 6 generations and a significant increase in the CSC markers ALDH1A1 and CD133 were seen in the PDX treated with carboplatin and paclitaxel for 4 weeks [24]. After dissociating cells from both the chemoresistant and the chemosensitive (parental) PDX model and plating cells both in tissue culture treated adherent conditions and in low attachment plates with stem-cell media, we treated all 4

types of cells with niclosamide, analog 11, or analog 32. In adherent conditions, the PDX#127-Resistant (127R) and PDX#127-Sensitive (127S) cells had similar sensitivity to all 3 agents (Fig 5A). When cells were grown in tumorsphere conditions, both niclosamide and analog 11 caused significantly more inhibition of proliferation (IC_{50} < 0.1) to the 127R chemoresistant cells compared to the 127S cells (IC_{50} < 1) (Fig 5B). This assay confirms our prior observations that niclosamide is able to preferentially target chemoresistant cells.

DISCUSSION

Niclosamide, an FDA approved anthelmintic drug, is an inhibitor of Wnt co-receptor LRP6 and suppresses both the Wnt/β-catenin pathways and the mTOR/STAT3 pathway. Niclosamide has been shown to be anti-proliferative against prostate, colorectal, lung, breast, and ovarian cancers, and myelogenous leukemia by inhibiting multiple pathways (Wnt/β-catenin, Notch, NF-kB, mTOR, and STAT3) and inducing mitochondrial uncoupling [13, 16, 25–33]. In ovarian cancer, it has been shown to specifically kill CSCs [20, 23]. However, niclosamide exerts its antiparasitic activity in the intestinal lumen and has poor bioavailability, which limits its potential application as an anticancer agent. Niclosamide is a nitroaromatic coumpound, and the presence of a nitro group (NO2) in a compound can induce metabolic instability and serous toxicity. In the present study, we use two niclosamide analogs that we designed, synthesized, and biologically evaluated. In analog 11, we replaced the NO2 group in the anilide portion with another electron withdrawing group, trifluoromethyl (CF3). The CF3 group, which is widely used in current drug design, is metabolically more stable than the NO2 group. In analog 32, the salicyl portion was modified. Both analogs showed similar biological

activity as niclosamide. They suppressed LRP6 expression, inhibited both Wnt/β-catenin and mTOR/STAT3 signaling in ovarian cancer ascites cells and chemoresistant cell lines, and displayed potent in vitro anti-proliferative effects. This data suggests that the novel niclosamide analogs are promising candidates for cancer therapy.

Interactions between the Wnt/β-catenin and STAT3/mTOR signaling pathways play an important role in ovarian carcinogenesis [34, 35]. There is significant cross-talk between these signaling pathways. The binding of a Wnt ligand to one of the transmembrane receptors LRP5/6 or Frizzled leads to the stabilization and translocation of active β-catenin in the cytoplasm. Stabilization and nuclear translocation of β-catenin leads to transcriptional activation of several important TCF/LEF target genes including survivin and cyclin D1 which are also regulated by mTOR signaling [36, 37]. Prior studies have shown that activated β-catenin causes enhanced STAT3 mRNA and protein expression [38]. In addition, studies have shown that activation of the STAT3 pathway leads to nuclear accumulation of β-catenin, causing increased levels of survivin and cyclin D1 [39]. Survivin is an oncogene that allows cells to recover from DNA damage and may contribute to chemotherapy resistance and help cells evade apoptosis. Cyclin D1 (CCND1), the proto-oncogene, is a cell-cycle regulator, which controls transition from G1 to S phase and has been shown to be correlated with platinum resistance. Given the high frequency of developing platinum resistance in ovarian cancer, it is imperative that we find treatments that target pathways that lead to chemotherapy resistance. Mechanistically, we found that niclosamide and two novel niclosamide analogs inhibit the production of both the oncogene survivin and the proto-oncogene cyclin D1, which both contribute to chemoresistance.

About 2% of ovarian cancer cells survive initial chemotherapy [40]. These CSCs have increased tumorgenicity, lead to recurrence, metastatic spread and chemoresistance. Wnt/β-catenin, mTOR, and STAT3 pathways have all been implicated in CSCs. ALDH1A1 and CD133 are two well established ovarian cancer stem cell markers. Both niclosamide and analog 32 preferentially killed the CD133+ population, which demonstrates that these agents could specifically target the CSCs. Niclosamide's ability to target the chemoresistant CSCs was also seen in the PDX model, where more effective inhibition of proliferation was seen in the cells dissociated from the chemoresistant PDX model and grown in stem cell media on low attachment plates.

Andrews et al. described a study where human volunteers received a single oral dose of 2,000 mg of carbonyl-14c-labeled niclosamide and the serum concentration was measured and found to be 0.25 - 6.0 μg/ml, which is equivalent to 0.7-18 μM [21]. These values are below the known Cmax of niclosamide in humans of 18 μM. We show in the present study that niclosamide and analogs 11 and 32 inhibited ovarian cancer cell proliferation in vitro with IC50 values between 0.41 and 1.86 μM. Anti-proliferative effects were seen in established ovarian cancer cell lines, A2780ip2 and SKOV3ip1, as well as the chemoresistant A2780cp20 and SKOV3Trip2 cell lines, which are enriched for CSCs [41, 42]. Niclosamide and its analogs were able to reverse the resistance of A2780cp20 to carboplatin, suggesting that in women with platinum-resistant ovarian cancer these agents could reverse chemoresistance.

ATPlite assay was initially performed to evaluate the cytotoxicity of niclosamide and its analogs, but the ATP assay is an indirect measure of cell number based on constant ATP. The results could have been confounded by niclosamide's well known

effect on oxidative phosphorylation in the mitochondria. Therefore, we used the trypan blue exclusion test in an effort to more accurately measure inhibition of proliferation. In order to better understand the anti-proliferative effect of the drugs, cell cycle arrest assay by flow cytometry and apoptosis ELISA assays were performed. Cell cycle arrest was seen at the G1 phase, which could be partially due to the decrease in the Wnt/β-catenin target gene, cyclin D1. While some amount of apoptosis was observed, this could be concentration dependent, contributing to only a small fraction of the biological response. The ATP level changes in the different cell types is likely a result of cell cycle arrest rather than cell kill.

Clinical trials are evaluating mTOR inhibitors, Wnt inhibitors, and STAT3 inhibitors in ovarian cancer and other solid tumors. Unfortunately, despite biological rationale and encouraging activity in preclinical models, trials of mTOR inhibitors in EOC have demonstrated disappointing results [43]. The Wnt/β-catenin and STAT3/mTOR pathways interact with each other and a number of other intracellular signaling networks, allowing for treatment escape when any single pathway inhibitor is used. Given the cross-talk between pathways and the adaptive capacity of cancer cells to evade targeted therapy, drug combinations are increasingly being investigated to abrogate both primary and acquired resistance to pathway specific targeted therapy. There has been evidence that aberrant expression of the Wnt pathway sensitizes cancer cells to an mTOR inhibitor; therefore, the use of a drug that targets both pathways would be even more effective than a single pathway inhibitor. Similarly, evasion from mTOR pathway inhibition could be due to activation and upregulation of the STAT3 pathway. The downside to multiple drug combinations is that these regimens can be toxic and difficult

to simultaneously administer. A single agent which targets multiple pathways and has an existing safety profile, such as niclosamide is promising.

Drug repurposing is an emerging approach for identifying new indications for existing drugs. Niclosamide is an example where drug repurposing has uncovered an existing drug that targets molecular pathways involved in carcinogenesis. Niclosamide is well-tolerated in humans and an oral dose of 2,000 mg of niclosamide reaches blood concentrations in humans that caused significant cancer cell anti-proliferative activity in chemoresistant cells [21]. Additional in vivo studies need to be performed to further evaluate the bioavailability and anti-tumor effects of analog 11 and analog 32.

Currently, niclosamide is being evaluated in a clinical trial with enzalutamide in treating patients with androgen receptor positive, castration-resistant, metastatic prostate cancer (NCT02532114). In addition, there are two clinical trials registered on www.clinicaltrials.gov for the treatment of colon cancer by niclosamide (NCT02687009, NCT02519582) but neither are open yet for participant recruitment. Our findings suggest that niclosamide has antitumor activity for ovarian CSCs through the inhibition of multiple altered cellular pathways associated with metastasis and cancer recurrence. The observation that niclosamide can target the Wnt, mTOR and STAT3 pathways and reverse platinum resistance, provides compelling evidence to consider clinical studies with niclosamide in patients with ovarian cancer. In conclusion, niclosamide has a novel mechanism as an antineoplastic agent that should be evaluated in ovarian cancer patients.

MATERIALS AND METHODS

Reagents and cell culture

Niclosamide was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in DMSO to create a 4.8 mM stock solution, which was stored at 4°C. Analogs 11 and 32 were synthesized in the laboratory of Dr. Pui-Kai Li at The Ohio State University (Columbus, OH), dissolved in DMSO and stored at concentrations of 10 mM at -20°C. A schematic is shown in Figure Figure11 of the Haygood et al. manuscript [12]. Carboplatin was purchased from Sigma-Aldrich (St. Louis, MO), dissolved in water at concentration of 25 mM, and stored at 4°C. Paclitaxel was purchased from UAB hospital pharmacy.

The ovarian cancer cell lines A2780ip2, A2780cp20, and SKOV3TRip2 were acquired courtesy of Dr. Charles N Landen. SKOV3ip1 was acquired from the American Type Culture Collection (Manasses, VA). The cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Atlanta Biologicals, Fowery Branch, GA). A2780cp20 (platinum- and taxane-resistant), SKOV3TRip2 (taxane-resistant), were generated by sequential exposure to increasing concentrations of chemotherapy [44]. SKOV3TRip2 was maintained with the addition of 150 ng/ml of paclitaxel. All cell lines were routinely screened for Mycoplasma species (GenProbe detection kit; Fisher, Itasca, IL) with experiments performed at 70–80% confluent cultures. Purity of cell lines was confirmed with STR genomic analysis, and only cells less than 20 passages from stocks were used in experiments.

Specimen collection and processing

Under IRB approval at UAB, all patients who were suspected to have ovarian cancer and scheduled to undergo surgery were consented for this study. Ascites fluid was collected at the time of laparotomy, diagnostic laparoscopy, or paracentesis. Cells were isolated from the ascites fluid via centrifugation at an initial 1000 rpm × 5 minutes, with serial spins at lower speeds (900–500 rpm), and finally at 400 rpm to remove red blood cells. Cell pellets were collected and washed in PBS, plated in ultra-low-attachment T-75 cm2 flasks (Corning Costar, Corning, NY) and incubated in X-vivo media (Lonza, Walkersville, MD) supplemented with 5 μg/mL insulin, and 20 ng/mL epidermal growth factor (PeproTech, Rocky Hill, NJ) in 37°C atmosphere and 5% CO2. Cells were cultured for 24 – 48 h and tumorspheres were then collected and stored at 10% DMSO and FBS for future analysis. In all samples, a board-certified gynecologic pathologist confirmed the cells to be ovarian cancer cells. PDX models 127S and 127R were developed in the laboratory of Dr. Landen. The PDX tissue was implanted in SCID mice. Tumors were harvested after reaching 15 mm x15 mm and were dissociated using Miltenyi Biotec Inc. (San Diego, CA) tissue homogenizer and tissue dissociation kits according to manufacturer's mouse tumor dissociation protocol. Dissociated tumors cells were plated in 96 well plates and analyzed by inhibition of proliferation assays after niclosamide or analog treatments.

Viability assays

Cells from ovarian cancer cell lines were were plated in 96 well plates, 2000 cells per well, and exposed to increasing concentrations of niclosamide, analog 11, or analog

32 alone or in combination with carboplatin in triplicate. The cells were lysed after 48 h and assessed for viability by using ATPlite luminescence-based assay (PerkinElmer, Waltham, MA) according to the manufacturer's protocol. For the trypan blue exclusion method, ovarian cancer cell lines were seeded in 6 well plates at 8000 cells / well and treated with increasing concentrations of niclosamide. The cells were harvested at 0 h, 24 h, 48 h and 72 h and were counted for live cells.

Western blot analysis

Cells from ovarian cancer cell lines or tumorspheres from ascites cells were seeded 1 million per well in 6 well plates and treated with niclosamide, analog 11, and analog 32 at the concentrations of 0.5 μM, 1 μM, 2 μM. Following 24 h, the cells were lysed in RIPA buffer supplemented with protease and phosphatase inhibitors and PMSF. Protein concentrations were determined with the BCA Protein Assay Kit (Pierce). Immunoblot analysis was carried out by standard techniques previously described [12]. Equal quantities of protein were subjected to SDS–PAGE under reducing conditions. Following transfer to immobilon-P membrane, successive incubations with anti-phosphorylated-LRP6, anti-LRP6, anti-total and free β-catenin, anti-survivin, anti-cyclin D1,4E-BP1, phosphor 4E-BP1, STAT-3, phosphor STAT-3, 70S6K, phospho70S6K, S6, phosphor S6 or anti-actin, and horseradish peroxidase-conjugated secondary antibody were carried out for 60 –120 minutes at room temperature (Supplementary Table S3). The immunoreactive proteins were quantified using the ECL system (PerkinElmer). Films showing immunoreactive bands were scanned by Kodak Digital Science DC120 Zoom Digital Camera (Kodak, Rochester, NY).

TOPflash luciferase reporter assay

Cells from ovarian cancer cell lines were seeded 40,000 per 50 μL in 96 well plates. Following 24 h, cells were transfected with 200 ng of TCF/LEF luciferase reporter (TOPFlash) (plasmid courtesy of Dr. Randall Moon, Upstate Biotechnology, Lake Placid, NY). Cells were transfected using Lipofectamine TM 2000 (Invitrogen, Carlsbad, CA) in Opti-MEM (Gibco/Invitrogen) per the manufacturer's instructions. After 6 h, cells were treated with 1 μM niclosamide, analog 11, or analog 32 and assayed for luciferase activity with or without Wnt3a ligand 24 h post-treatment. Luciferase activity was measured using a Turner 20/20 luminometer (Promega, Madison, WI) and was normalized to the total protein concentration as reported previously [23]. The luciferase activity was normalized to untreated control and represented as the mean ± SE for a minimum of 3 replicates. Recombinant Human Wnt-3a Protein (100 ng/ml) R&D Systems Inc. Minneapolis, MN (Catalog #5036-WN-010) was used to stimulate β-catenin driven Wnt signaling.

Cell cycle analysis

For cell cycle analysis, cells were treated with vehicle, 1μM, 2 μM and 4 μM doses of niclosamide for 48 h, trypsinized, and fixed in 100% ethanol overnight. The dead floating cells were also collected and combined with live cells for staining. Cells were then centrifuged, washed in PBS, and re-suspended in PBS containing 0.1% Triton X-100 (v/v), 200 μg/mL DNase-free RNase A and 20 μg/mL propidium iodide (PI). PI fluorescence was assessed by flow cytometry and the percentage of cells in G0/G1, S and

G2/M phases was calculated by the cell cycle analysis module for Flow Cytometry Analysis Software (FlowJo v.7.6.1, Ashland, OR).

Apoptosis detection

Apoptosis was assessed with cell death detection ELISA kit purchased from Sigma Aldrich (St. Louis, MO). Cells were treated with vehicle, 1μM, 2 μM and 4μM of niclosamide for 48 h. The spent medium containing floating cells was saved and kept on ice. The adherent cells were collected by gentle trypsinization and were combined with the floaters for pelleting by centrifugation. After gentle lysis of the cells with the buffer provided with the detection kit, the cell lysate was used for the ELISA test. The results were normalized by the protein content obtained from parallel plates with the cells being lysed using the buffer as described above for Western blotting. Apoptosis was detected by presence of histone associated DNA fragments that can be found in cell cytoplasm several hours before plasma membrane breakdown.

CD133 expression in ovarian cancer cell lines

A2780cp20 cells were harvested after 48 h of treatment with niclosamide, analog 11, or analog 32 at 2 μM, or paclitaxel 70 nM, Carboplatin 380 μM. Cells were stained with CD133 antibody for 30 min on ice and were analyzed for CD133 expression by flow cytometry.

Statistical analysis

Statistical significance for TOPflash, cell cycle analysis, and ELISA were determined by Student's t-test and $P < 0.05$ was considered significant. To test differences in treatment effects, ANOVAs and Tukey tests were performed. In cases when comparisons had to be made for greater than two groups, ANOVAs were run. We followed with Tukey tests to compare the means of every treatment to the means of every other treatment to identify differences between any two means. Calculations were performed using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA).

The IC50 (half maximum inhibitory concentration) was defined as the log10 of the niclosamide or analog concentration producing 50% reduction in ATP levels (in counts per second) compared with the untreated ascites or cells. Cell viability was measured using a ratio of ATP levels for treated tumorspheres or cell lines to untreated controls (percent control). The combination index (CI) for the dose–effect relationship of niclosamide or the analogs and carboplatin were calculated based on the multiple drug-effect equation of Chou–Talalay for calculation of synergy [45, 46]. $CI = DA/ICx,A + DB/ICX,B$ where ICX,A and ICX,B are concentrations of drugs producing X % inhibition for each respective drug alone, and DA and DB are concentrations of each drug in the mixture that yield X % inhibition. The CI curve or modified isobologram is generated by plotting CI vs. X, ranging from 0 to 100%. Drug interactions are readily identified at any level of inhibition. The resulting CI theorem offers quantitative definition for additive effect ($CI = 1$), synergism ($CI < 1$), and antagonism ($CI > 1$). The quantitative diagnostic plot was generated with Calcusyn software version 2.0 (Biosoft,

Ferguson, MO). All data represents an average of at least four replicates. Error bars represent mean ± SE as indicated in the figure legends.

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Table 1: IC_{50} doses of niclosamide, analog 11 and analog 32 on ovarian cancer cell lines

IC_{50} µM	A2780ip2	A2780cp20	SKOV3ip1	SKOV3TRip2
Niclosamide	0.59	0.56	1.83	1.13
Analog 11	0.55	0.41	0.8	0.83
Analog 32	0.65	0.75	1.86	1.66

**A2780ip2, A2780cp20, SKOV3ip1, SKOV3TRip2 cancer cell lines were treated with niclosamide, analog 11 or 32 (0.1- 4 µM) for 48 h. Cells were analyzed for viability using ATPlite assay. The niclosamide IC_{50} (half maximum inhibitory concentration) was defined as the $\log_{10}$ concentration generating a 50% reduction in ATP levels when compared with the untreated control.

Table 2: Cell count by trypan blue exclusion method

SKOV3TRip2	Untreated control	1 µM	2 µM	4 µM
Plating time	8000	8000	8000	8000
0 h	12150	12150	12150	12150
24 h	21100	16175	13350	13050
48 h	47600	30600	16300	12800
72 h	51250	36250	18366	12166

A2780cp20	Untreated control	1 µM	2 µM	4 µM
Plating time	8000	8000	8000	8000
0 h	20000	20000	20000	20000
24 h	47291	28958	20416	15000
48 h	129250	53750	14000	8250
72 h	346050	88600	26133	7400

**A2780cp20, SKOV3TRip2 cell lines were plated in 12 well plates and treated with niclosamide at indicated concentrations. Cell viability was measured by trypan blue exclusion method.

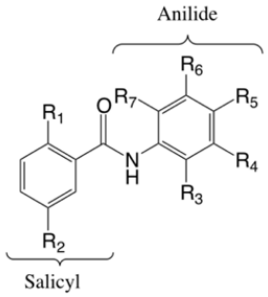
Table 1. IC_{50} doses of niclosamide, analog 11 and analog 32 on ovarian cancer cell lines.

Table 2. Cell count by trypan blue exclusion method.

Table 3: Antibody Information for Western Blotting

Number	Antibody	Concentration	Company Name and Catalog Number
1	Stat3	1:1000	Cell Signaling # 4904
2	P(Tyro705) stat3	1:500	Cell Signaling # 9131
3	P70S6K	1:1000	Cell Signaling # 9202
4	P(Thr389)P70-70SK	1:1000	Cell Signaling # 9205
5	S6	1:2000	Cell Signaling # 2217
6	P(Ser235/236)S6	1:2000	Cell Signaling # 4857
7	LRP6	1:1000	Cell Signaling # 3395
8	4E-BP1	1:1000	Cell Signaling # 9644
9	P4E-BP1	1:1000	Cell Signaling # 13443
10	Cyclin D1	1:1000	Cell Signaling # 2978
11	Survivin	1:1000	Cell Signaling # 2808

Table 4: Structures of niclosamide and analog 11 and 32

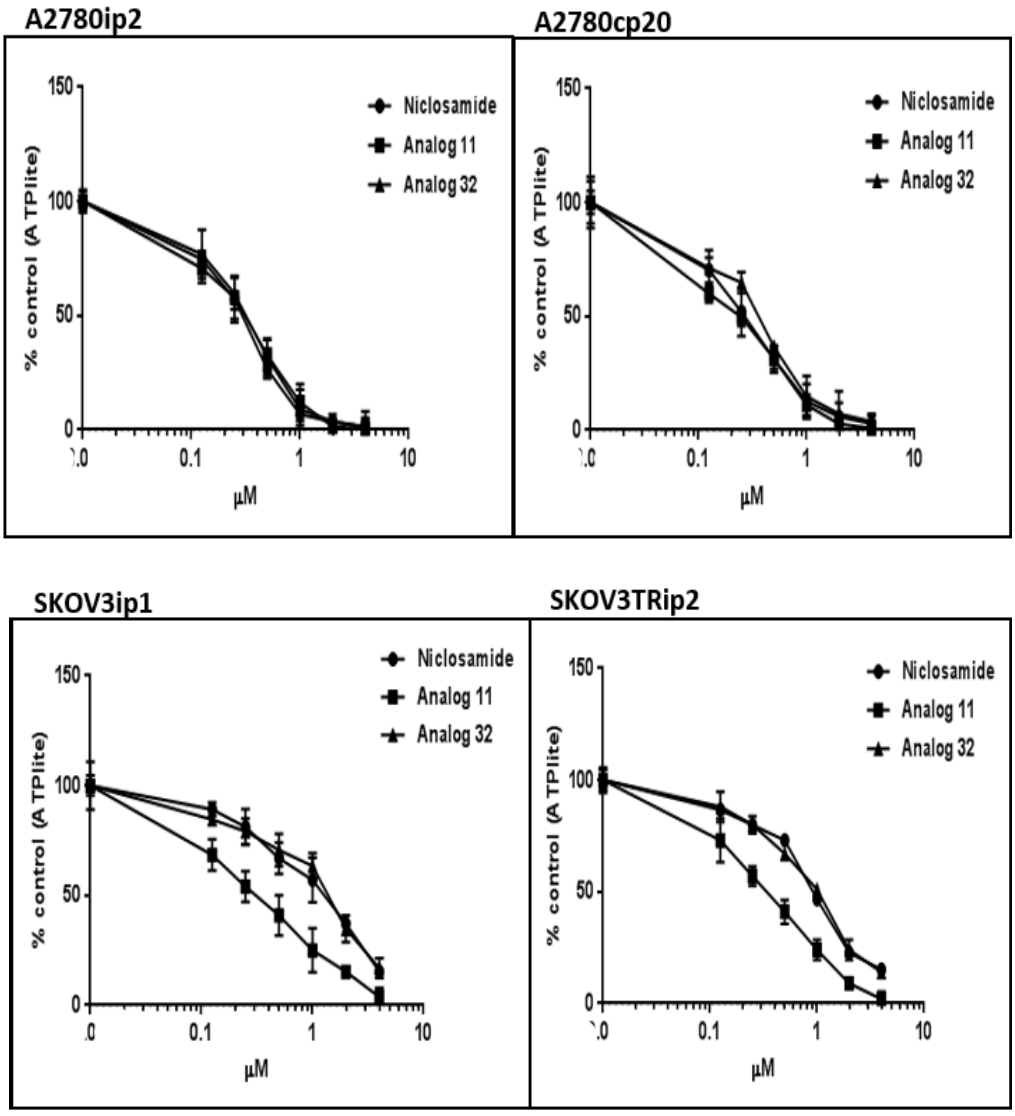


Compound	R^1	R^2	R^3	R^4	R^5	R^6	R7
Niclosamide	OH	Cl	Cl	H	NO_2	H	H
Analog 11	OH	Cl	H	H	CF_3	H	H
Analog 32	$OONH_2$	Cl	Cl	H	NO_2	H	H

Table 3. Antibody information for Western blotting.

Table 4. Structures of niclosamide and analogs 11 and 32.

A


A2780ip2
A2780cp20
SKOV3ip1
SKOV3TRip2
Niclosamide
Analog 11
Analog 32
% control (ATPlite)
150
100
50
0
0.1
1
10
µM

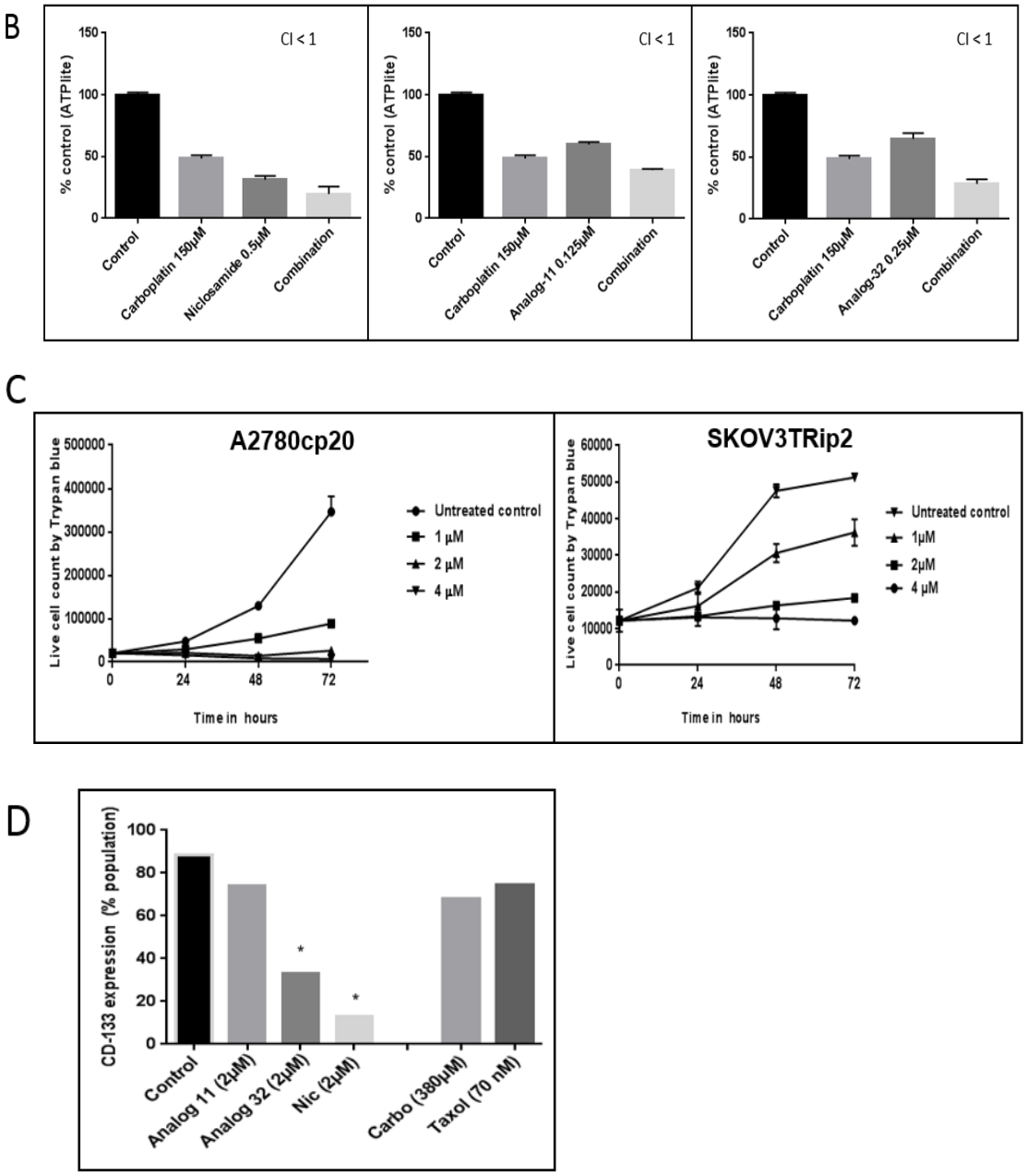
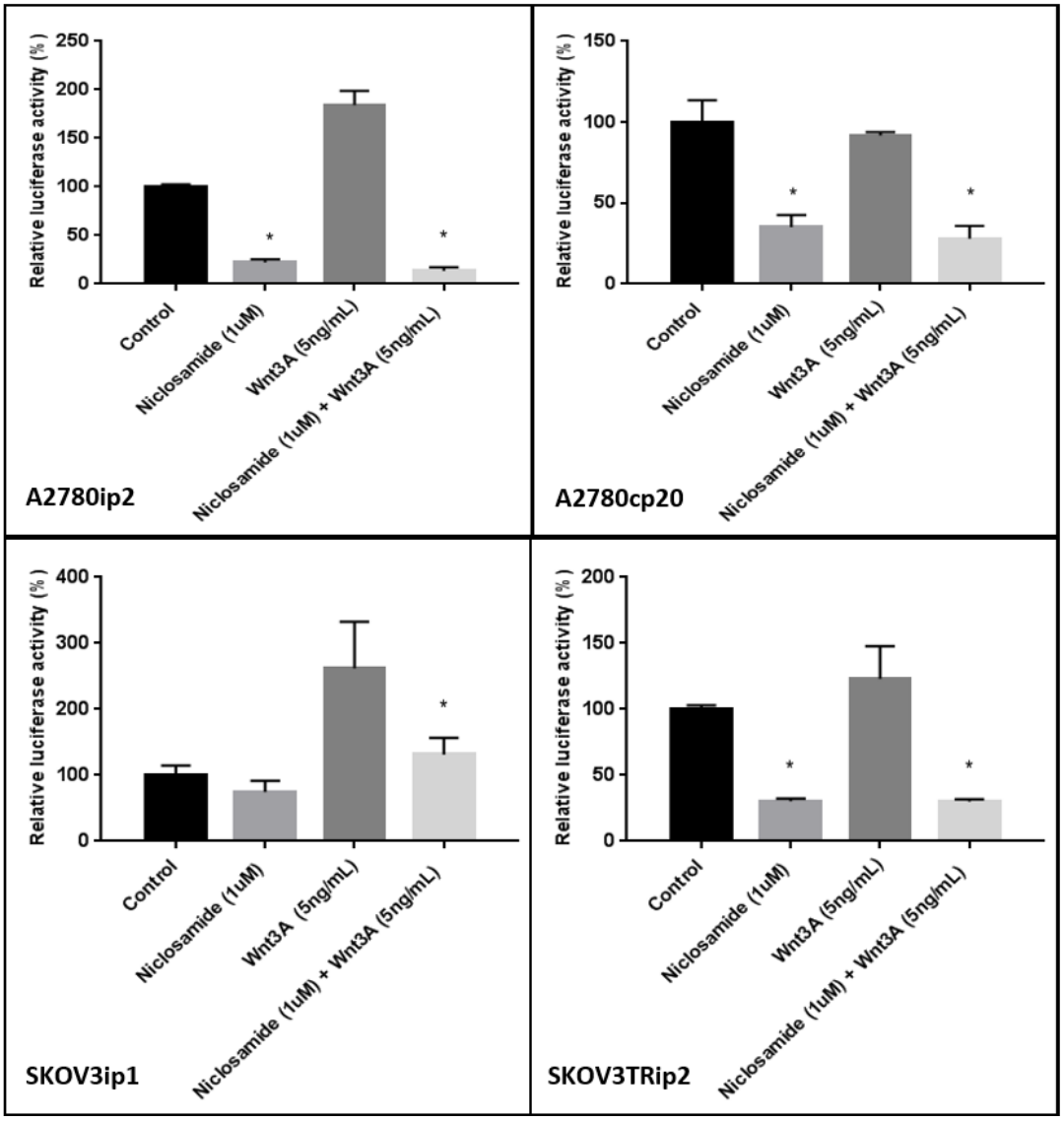


Figure 1 Anti-proliferative effects of niclosamide, and its analogs, as single agents and in combination with chemotherapy and expression of CD133 post treatment. (A) A2780ip2, A2780cp20, SKOV3ip1, SKOV3TRip2 cancer cell lines were treated with niclosamide, analog 11 or 32 (0.1- 4 μM) for 48 h. Level of ATP in the treated cells compared to the untreated cells were analyzed using ATPlite assay. (B) A2780cp20 cells were treated concurrently with niclosamide or analogs in combination with carboplatin at indicated concentrations for 48 h. A combination index (CI) was calculated where CI <1 is synergistic. (C) A2780cp20, SKOV3TRip2 cell lines were plated in 12 well plates and treated with niclosamide at indicated concentrations. Cell viability was measured by trypan blue exclusion method. All experiments were repeated 3 times. (D) A2780cp20

cells were treated with niclosamide or analogs (2 μM), or IC50 doses of chemotherapy for 48 h and analyzed for CD133 expression by flow cytometry. Using student's t-test, *P < .05. All experiments were repeated 3 times. Data are represented as mean ± SD. Statistical analyses were performed using one-way ANOVA with application of Tukey's post test, P < .05 for all figures in (B).

A


Relative luciferase activity (%)
250
200
150
100
50
0
*
*
Control
Niclosamide (1uM)
Wnt3A (5ng/mL)
Niclosamide (1uM) + Wnt3A (5ng/mL)
A2780ip2
Relative luciferase activity (%)
150
100
50
0
*
*
Control
Niclosamide (1uM)
Wnt3A (5ng/mL)
Niclosamide (1uM) + Wnt3A (5ng/mL)
A2780cp20
Relative luciferase activity (%)
400
300
200
100
0
*
Control
Niclosamide (1uM)
Wnt3A (5ng/mL)
Niclosamide (1uM) + Wnt3A (5ng/mL)
SKOV3ip1
Relative luciferase activity (%)
200
150
100
50
0
*
*
Control
Niclosamide (1uM)
Wnt3A (5ng/mL)
Niclosamide (1uM) + Wnt3A (5ng/mL)
SKOV3TRip2

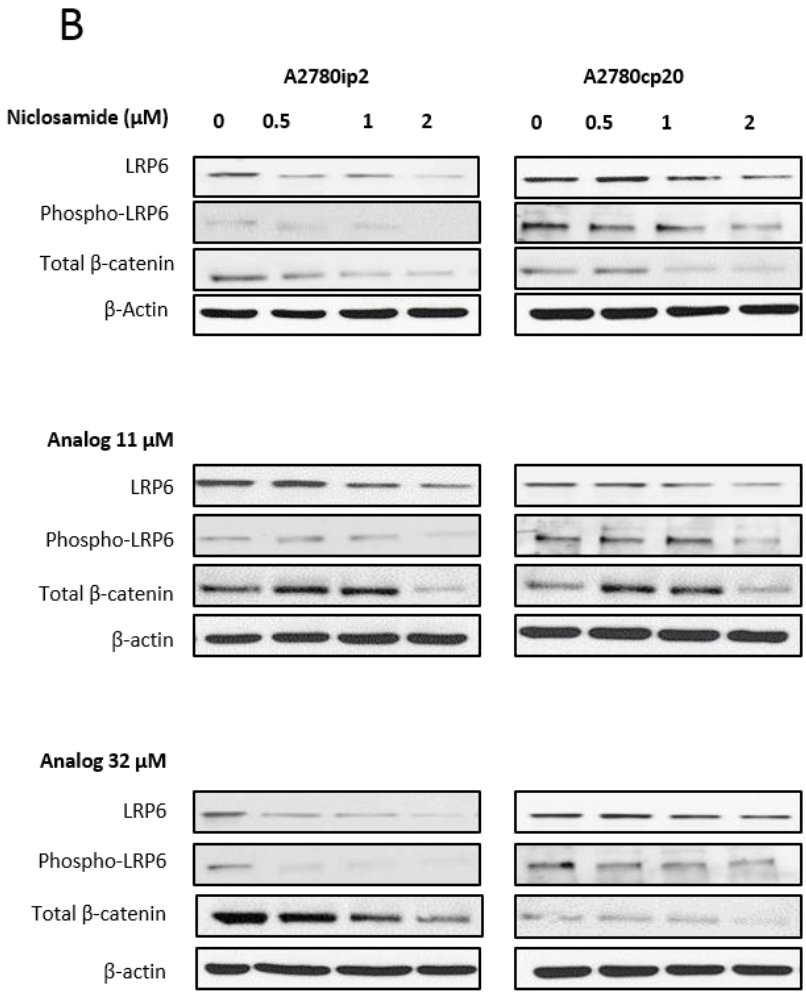


Figure 2 Wnt/β-Catenin specific inhibition of ovarian cancer cell lines. (A) A2780ip2, A2780cp20, SKOV3ip1 and SKOV3TRip2 cells in 24 well plates were treated with niclosamide and or Wnt3A, along with TOPflash construct and β-galactosidase-expressing vector in each well. After being incubated for 24 h, cells were analyzed for Wnt signaling. **(B)** A2780ip2 and A2780cp20 cells were treated in 6 well plates with niclosamide and analogs at indicated concentrations for 24 h. The levels of LRP6 and phospo-LRP6, total β-catenin, were examined by western blot. All experiments were repeated 3 times. Data are represented as mean ± SD. Statistical analyses were performed by using student's t-tests, * $P < 0.05$ when niclosamide group was compared to untreated control and niclosamide with Wnt3A group was compared to Wnt3A alone.

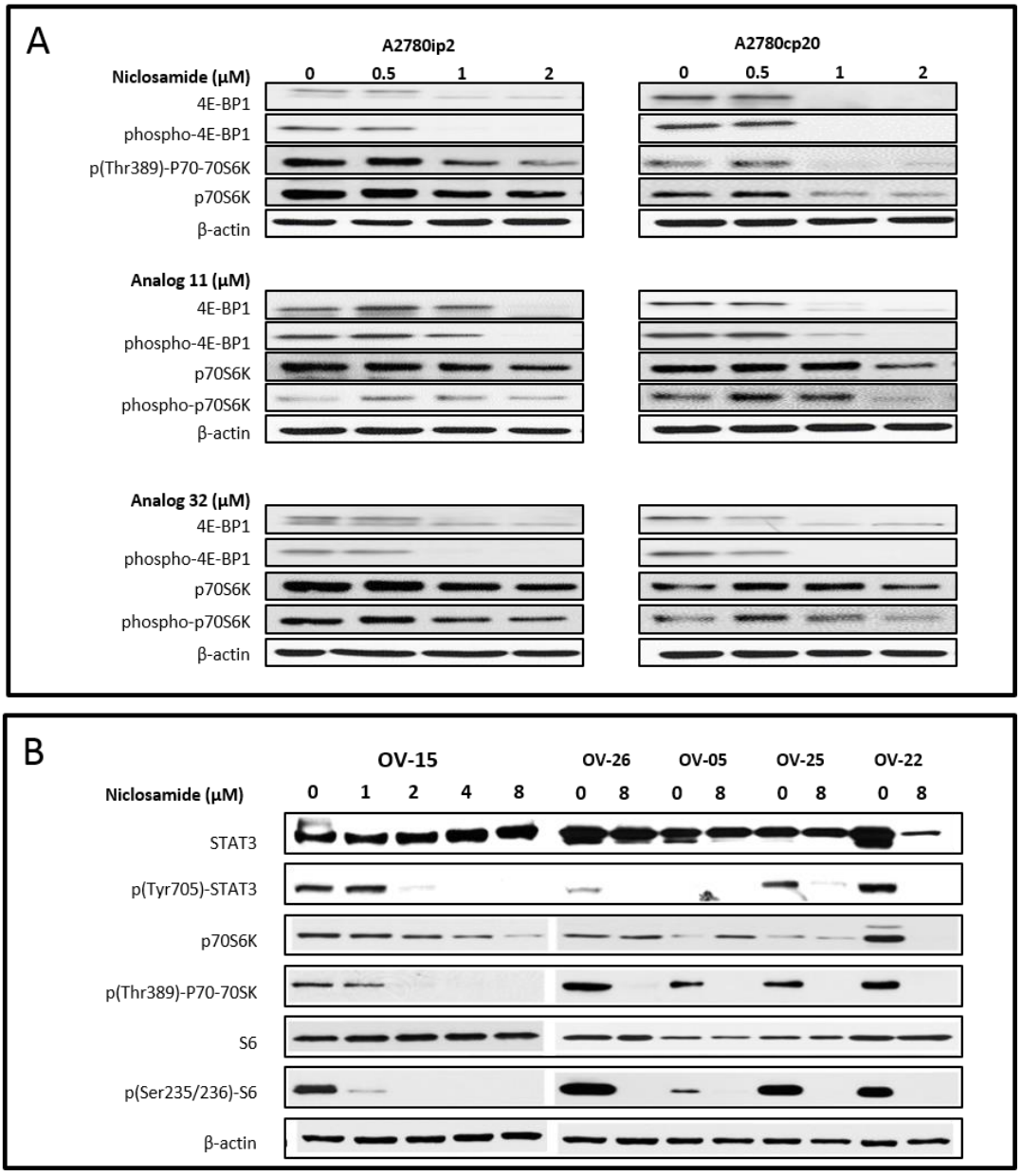
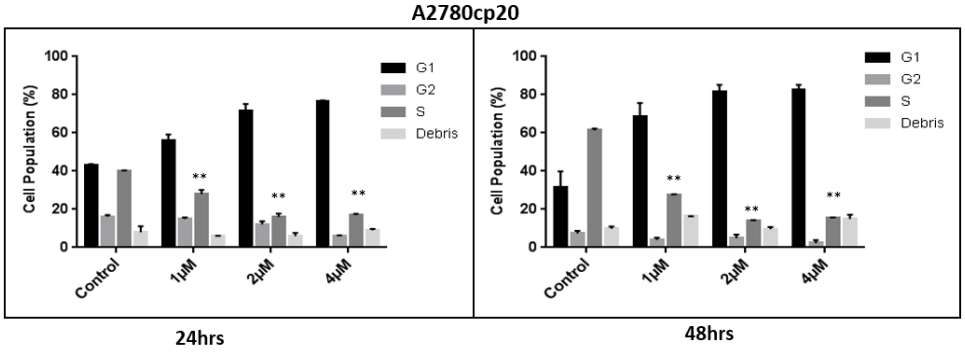
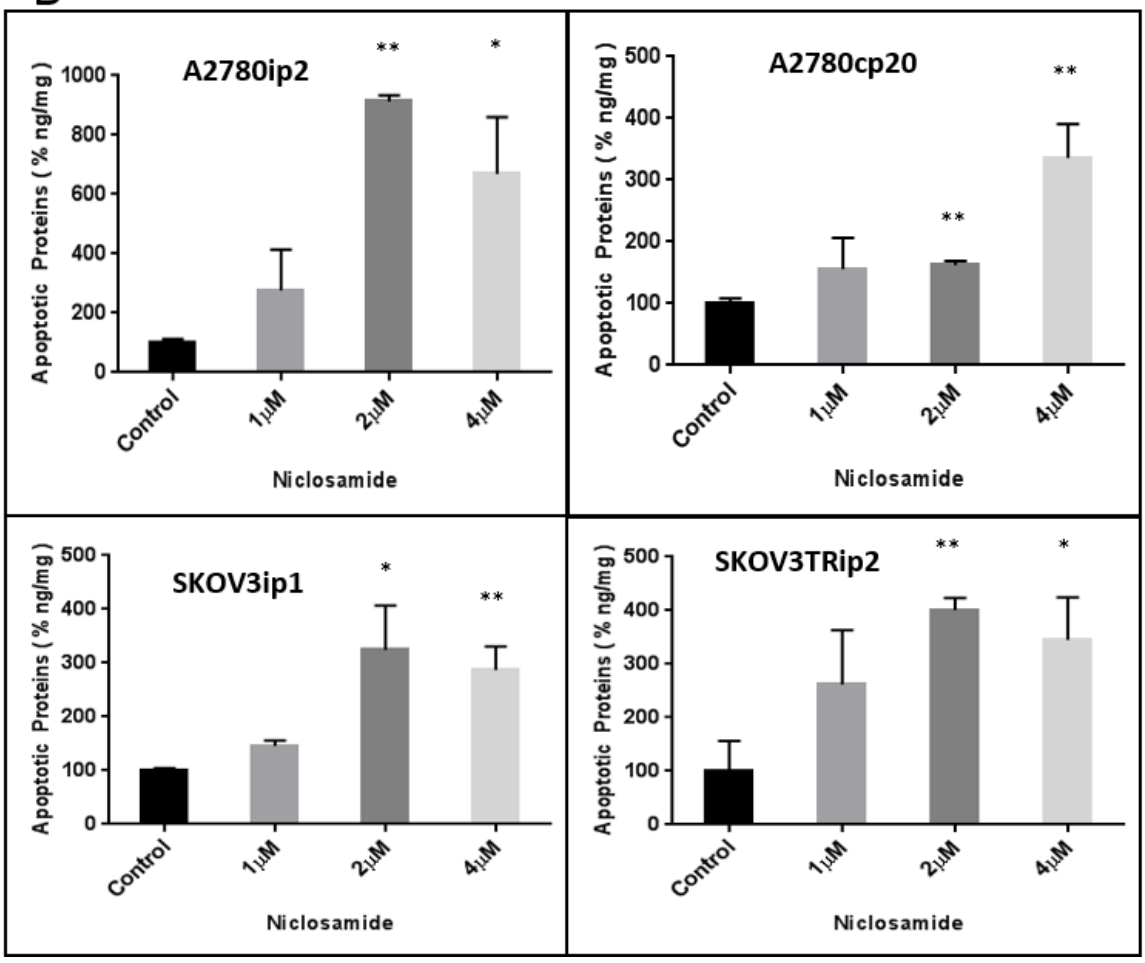


Figure 3 mTOR/STAT3 inhibition of ovarian cancer cell lines and patient samples **(A)** A2780ip2, A2780cp20 cell lines were plated in 6 well plates and were treated with niclosamide and analogs 11 and 32 at indicated concentrations for 24 h. Treated lysates were examined for the levels of mTOR pathway proteins (4E-BP1, phospho-4E-BP1, p(Thr389)-P70-70S6K, p70S6K). **(B)** Acites cells from ovarian cancer patients were plated in 6 well plates and were treated with niclosamide at indicated concentrations for 24 h. Treated lysates were examined for the levels of STAT3 pathway proteins (p(Tyr705)-STAT3 and STAT3) and mTOR pathway proteins (p(Thr389)-p70-70SK, p70S6K, p(Ser235/236)-S6, and S6).

A


A2780cp20
Cell Population (%)
G1
G2
S
Debris
Control
1μM
2μM
4μM
24hrs
48hrs

B


A2780ip2
A2780cp20
SKOV3ip1
SKOV3TRip2
Apoptotic Proteins (% ng/mg)
Control
1μM
2μM
4μM
Niclosamide

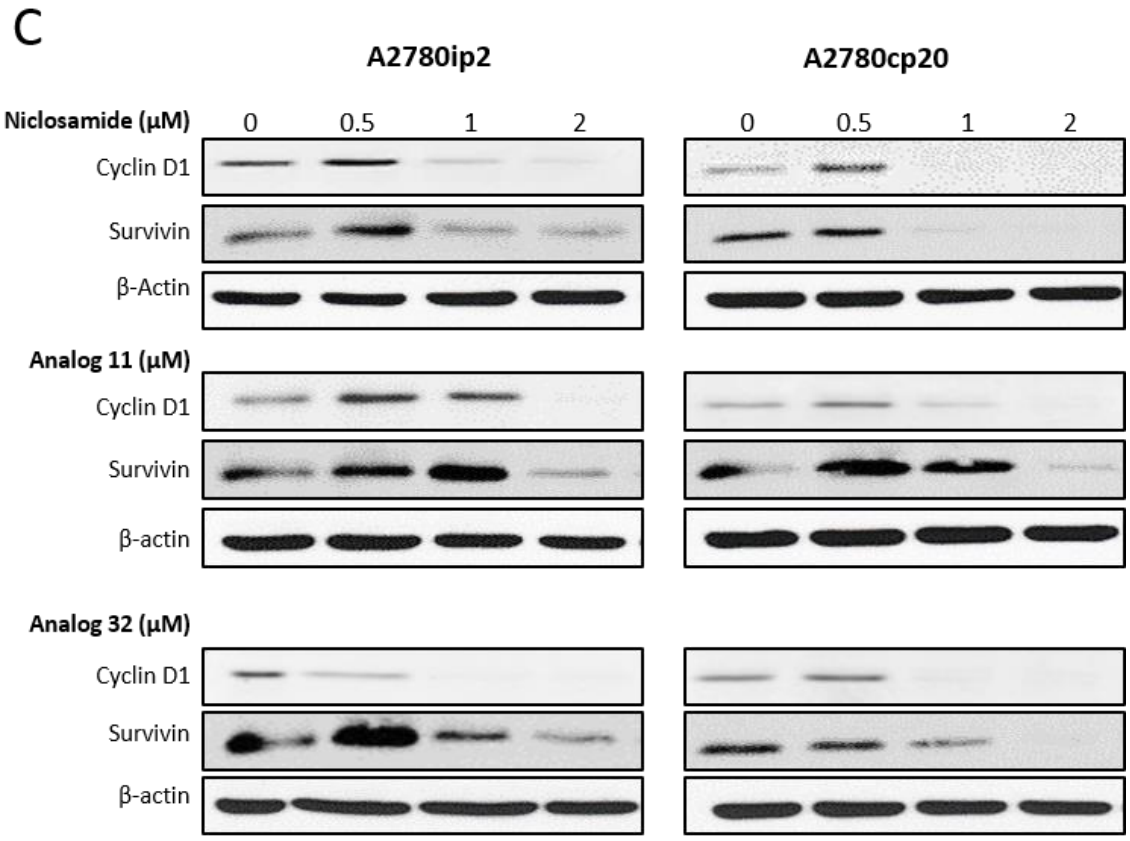


Figure 4 Cell cycle arrest by niclosamide and effect of niclosamide on cancer cell apoptosis and proliferation. (A) A2780cp20 cell line was plated in 12 well plates and treated with indicated concentrations of niclosamide. Cells were stained with PI as described in Materials and Methods. Percentages of cells in different phases of the cell cycle were determined by flow cytometry at 24 and 48 h. (**B**) A2780ip2, A2780cp20, SKOV3ip1, SKOV3TRip2 cancer cell lines were treated with niclosamide at indicated concentrations for 48 h. Floating and attached cells were combined for apoptosis detection by Cell Death ELISA kit for histone–associated DNA fragments as described in Materials and Methods. **(C)** A2780ip2, A2780cp20 cell lines were treated with indicated concentrations of niclosamide. Lysates from treated cell lines were analyzed for survivin and cyclin D1. Data are represented as mean ± SD. Statistical analyses were performed by using student's t-tests, *P<.05, **P<.005 for figures (A) and (B).

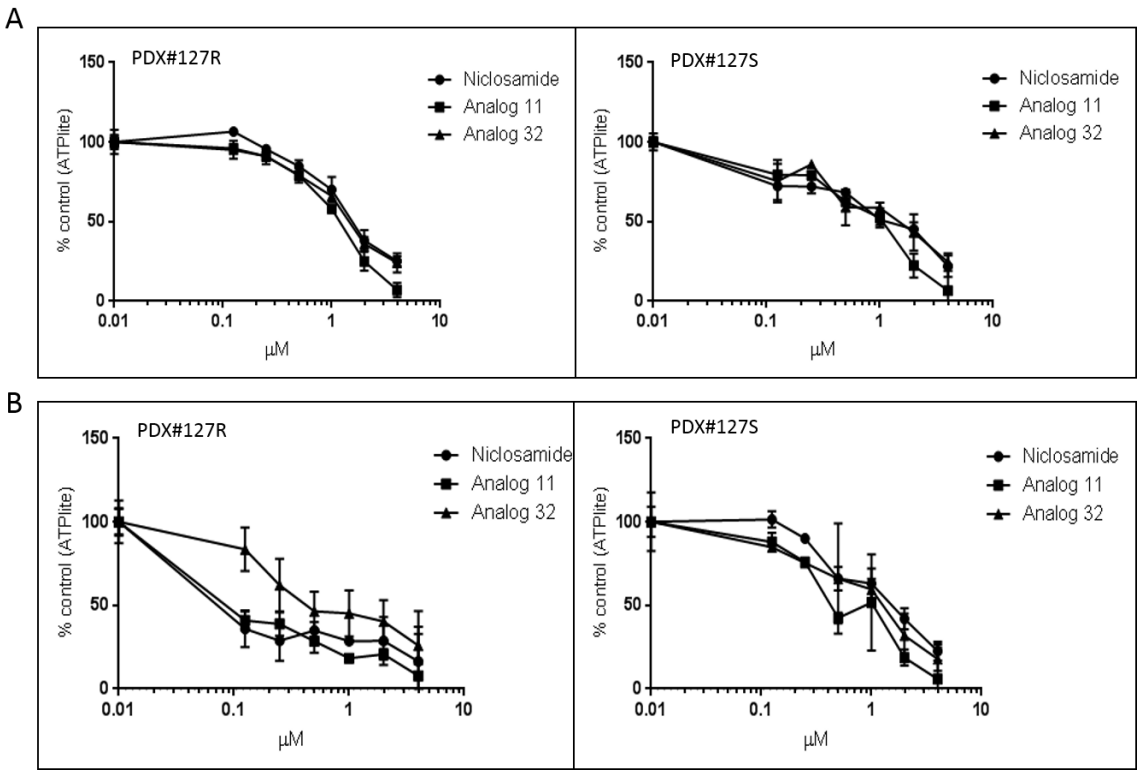
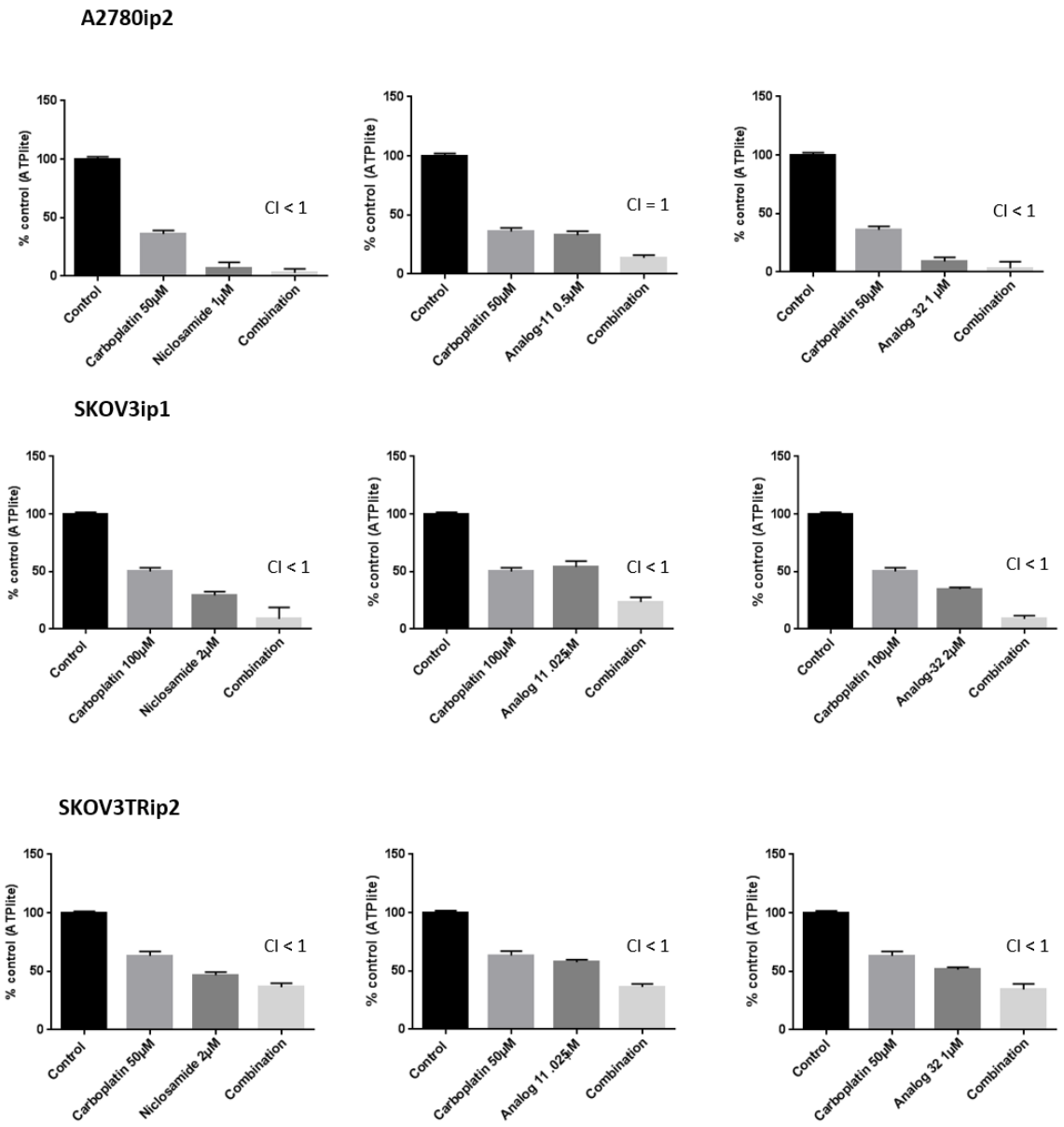
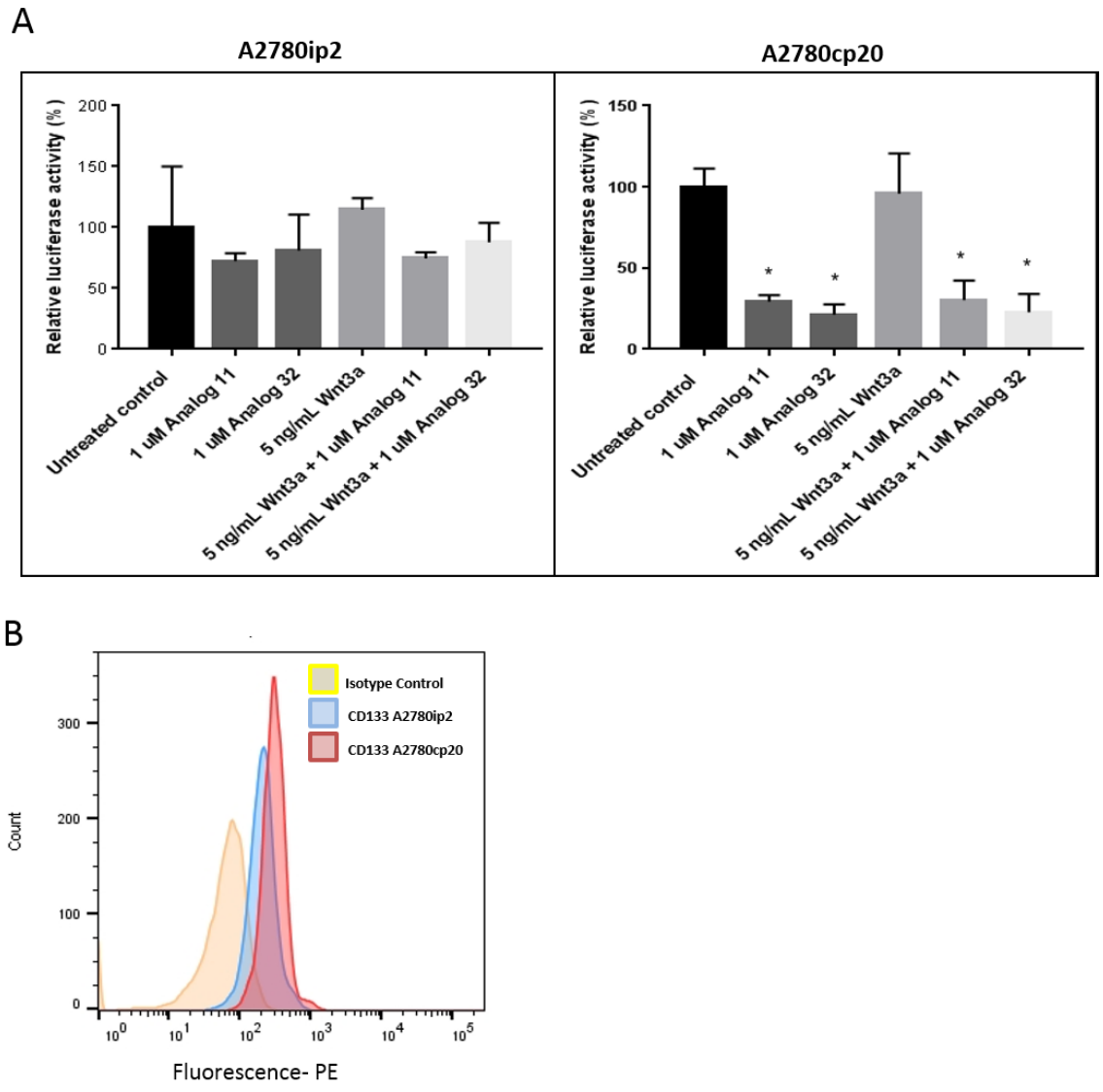


Figure 5 Anti-proliferative effect of niclosamide and analogs 11 and 32 on PDX mouse model cells. PDX models 127 R (resistant) and 127 S (sensitive) were dissociated to single cell suspension and plated in 96 well plates. **(A)** PDX cells were plated in tissue culture treated plates in 10% FBS + DMEM media. **(B)** PDX cells were plated in low attachment plates with serum free x-vivo media with added supplements to promote stem cell growth. Cells were treated with niclosamide or analogs at increasing concentrations. Viability of cells was analyzed by ATPlite assay. All experiments were repeated 3 times. Data are represented as mean ± SD.



Supplementary Fig 1. Combination treatment of ovarian cancer cell lines. A2780ip2, SKOV3ip1 and SKOV3TRip2 cells were treated concurrently with niclosamide or analogs in combination with carboplatin at indicated concentrations for 48 h. All experiments were repeated 3 times. Data are represented as mean ± SD. Statistical analyses were performed by using one-way ANOVA with application of Tukey's post test, $P < .05$ when compared to untreated control for all figures. A combination index (CI) was calculated where CI <1 is synergistic and CI = 1 is additive.



Supplementary Figure 2. TOPflash WNT activity and CD133 expression on A2780ip2 and A2780cp20 cells. (A) A2780ip2 and A2780cp20 cells were treated in 24 well plates with 1 µM analog 11 and/or analog 32, Wnt3A and TOPflash construct and β-galactosidase-expressing vector in each well for 24 h and analyzed for WNT signaling. **(B)** A2780ip2 and A280cp20 cells were stained for CD133 expression and compared to isotype control. Data are represented as mean ± SD. Statistical analyses were performed using student's t-test, *P < .05 when analog 11 or analog 32 group was compared to untreated control and analog 11 or analog 32 with Wnt3A group was compared to Wnt3A alone.

INHIBITION OF WNT/β-CATENIN SIGNALING BY NOVEL BENZIMIDAZOLES IN TRIPLE-NEGATIVE BREAST CANCER

by

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ABSTRACT

Wnt/β-catenin signaling is upregulated in triple-negative breast cancer (TNBC) compared to other breast cancer subtypes and normal tissues. Current Wnt/β-catenin inhibitors, such as niclosamide, target the pathway nonspecifically and exhibit poor pharmacokinetics/pharmacodynamics *in vivo*. Niclosamide targets other pathways, including mTOR, STAT3, and Notch. Novel benzimidazoles have been developed to inhibit Wnt/β-catenin signaling with greater specificity. The compounds SRI33576 and SRI35889 were discovered to produce more cytotoxicity in TNBC cell lines than in noncancerous cells. The agents also downregulated Wnt/β-catenin signaling mediators LRP6, cyclin D1, survivin, and nuclear active β-catenin. In addition, SRI33576 did not affect mTOR, STAT3, and Notch signaling in TNBC and noncancerous cells. SRI35889 inhibited mTOR signaling less in noncancerous than in cancerous cells, while not affecting STAT3 and Notch pathways. Compounds SRI32529, SRI35357, and SRI35361 were not selectively cytotoxic against TNBC cell lines compared to MCF10A cells. While SRI32529 inhibited Wnt/β-catenin signaling, the compound also mitigated mTOR, STAT3, and Notch signaling. SRI33576 and SRI35889 were identified as cytotoxic and selective inhibitors of Wnt/β-catenin signaling with therapeutic potential to treat TNBC in vivo.

INTRODUCTION

Of all cancers, breast cancer affects women the most with an incidence of about 12%. High morbidity of this disease is associated with triple-negative breast cancer (TNBC), which accounts for 10-20% of all breast cancers [1-3] The moniker of the

disease derives from lack of progesterone, estrogen, and HER2 receptors, commonly targeted by chemotherapy, hormone therapy, and HER2 antibody. As a result, these cancers exhibit drug resistance [4]. Known as the most aggressive breast cancer subtype, TNBC requires new targeted therapies to overcome resistance and lessen probability of relapse.

Underlying signaling characteristics of TNBC may be exploited for developing novel therapies. Wnt/β-catenin signaling drives tumorigenesis in a variety of cancers and contributes to progression of TNBC compared to that of other breast cancer subtypes [5-8]. Wnt/β-catenin signaling is activated upon binding of secreted cysteine-rich Wnt glycoproteins to LRP5/6 and Frizzled co-receptors, leading to inactivation of β-catenin-phosphorylating protein GSK3β. Lack of phosphorylation enables β-catenin to avoid ubiquitination and degradation, thus allowing the protein to translocate to the nucleus, bind to TCF/LEF transcription factors, and promote transcription of target genes, such as cyclin D1 and survivin, that drive tumorigenesis and cell proliferation [9-12].

Unlike colorectal cancers, of which 80-90% contain APC mutations, TNBC generally lacks mutations in Wnt/β-catenin pathway related proteins. Instead, pathway dysregulation is mediated by activation of β-catenin-mediated transcription [7, 13] and high expression of Wnt/β-catenin pathway mediators such as Frizzled [14, 15] and LRP6 [16]. Both autocrine and paracrine Wnt/β-catenin signaling are implicated in TNBC progression [17-19]. LRP6 surface receptor is overexpressed in triple-negative, ER-negative, and HER2-negative breast cancers. At the genetic level, LRP6 expression is more than 6-fold higher in 6 of 7 TNBC cell lines compared to noncancerous MCF10A cells [16]. At the protein level, LRP6 was overexpressed in 4 of 7 TNBC cell lines

compared to MCF10A cells. Little overlap was observed between tumor samples displaying higher LRP6 transcript and those with higher HER2 transcript, signifying that Wnt/β-catenin signaling is possibly an independent diagnostic indicator [16]. Inhibition of LRP6 expression and/or activity with Mesd, an LRP6 antagonist, in MDA-MB-231 cells decreased cell viability, proliferation, and colony formation [16]. In addition, inhibition of LRP6 activity led to decrease in migration and invasion of TNBC cells. Treatment with Mesd also decreased expression of S100A4, a Wnt/β-catenin signaling target and a contributor to cancer metastasis [20]. As a result, targeting LRP6 represents an appealing therapeutic strategy for TNBC. Blood of mice dosed with 5, 10, 20 mg/kg Mesd had the highest concentration after 2 h. Tissue distribution of Mesd was quick and widespread as the bioavailability averaged about 60% [16].

Novel small molecule inhibitors targeting Wnt/β-catenin signaling in cancers have been recently developed [21]. Appealing targets have included CREB-binding protein (CBP), a coactivator involved in β-catenin-mediated transcription by binding either β-catenin or TCF [22], and porcupine, a Wnt-modifying enzyme [23]. Currently, agents such as PRI-724, a CBP suppressor, and WNT974, a porcupine inhibitor, are undergoing investigation for use as sole agents in phase I clinical trials in TNBC patients [24]. Small molecule inhibitor CWP232228 suppressed β-catenin-mediated transcription in TNBC by mitigating β-catenin/TCF interaction, inhibiting stem cell proliferation and lowered tumor bulk [25]. The agent displayed minimal toxicity and no obvious clinical signs in animals. CWP232228, administered IV at dose of 200 mg/kg yielded blood concentration of 0.8 μg/mL for 7 hours [25]. Inhibitors such as XAV939 [26] and IWP-2 [27] have been shown to target Wnt/β-catenin activity via in vitro studies, although they exhibit

suboptimal pharmacokinetic and pharmacodynamic (PK/PD) characteristics. As a result, Wnt/β-catenin pathway inhibitors with improved PK/PD activities are required for more promising therapies in vivo.

Maintenance of cancer stem cells (CSCs), a subpopulation of cells involved in self-renewal and regeneration of tumors, has been associated with Wnt/β-catenin signaling. Breast CSCs, identified by CD44+/CD24- markers, have the capacity to generate tumors [28, 29]. High β-catenin expression correlates with the CD44+/CD24- phenotype [30]. In addition, suppression of WNT1 changes the phenotype and lessens tumor formation and cell migration [31]. Attenuation of Wnt/β-catenin signaling due to inhibition of protein kinase D1 (PKD1) decreased CSC characteristics in breast cancer cells [32].

High-throughput screening of more than 4,000 clinically approved compounds revealed the anthelmintic agent niclosamide as a potent Wnt/β-catenin signaling inhibitor with anticancer activity [33]. Currently undergoing clinical trials in colorectal and prostate cancers, niclosamide inhibits proliferation of various cancers at concentrations that also inhibit Wnt/β-catenin signaling activities [34, 35]. Niclosamide inhibited Wnt/β-catenin signaling in TNBC by inducing LRP6 degradation, enabling Frizzled1 internalization, and mitigating interaction between β-catenin and TCF [34-37]. Anthelmintic activities of niclosamide have been shown to influence ATP homeostasis and uncoupling of oxidative phosphorylation [38, 39]. Potentially, niclosamide may inhibit Wnt/β-catenin signaling and elicit anthelmintic effects via different mechanisms [40]. In addition, the drug has also been found to impact mTOR, STAT3, and Notch signaling in various cancers [41-46]. Our laboratory has previously shown niclosamide

affects both Wnt/β-catenin and STAT3 signaling pathways in bulk and stem TNBC cells [47]. Niclosamide has shown some efficacy after systemic administration against various cancers in animal studies, but the agent is not well absorbed from the gastrointestinal tract following oral administration as used for treatment of tapeworm, attributed to its weak metabolic stability (t1/2 = 29 minutes in rat liver microsomes) and solubility (1.6 μM, pH 7.4). Ideally, the pharmacokinetic t1/2 should be at least 60 minutes, requiring twice daily dosing.

Benzimidazole compounds (agents featuring fused benzene and imidazole rings) have been evaluated for anticancer effects [40, 48-50]. Due to the poor bioavailability of niclosamide, several benzimidazole compounds were developed by modifying the structure of niclosamide to improve in vivo potential. The amide group of niclosamide was constrained to generate the five-membered ring scaffold for benzimidazole analogs used in this study. A class of compounds, known as 2,5-disubstituted phenyl benzimidazoles, produced cytotoxic and Wnt/β-catenin-inhibiting effects. These compounds are characterized by substituents located in the 2 and 5 positions of the phenyl ring in benzimidazoles. The NO2 group in niclosamide, known to elicit toxic effects, was removed from most of the inhibitors. Since niclosamide was postulated to affect Wnt/β-catenin signaling separately from ATP homeostasis, Mook et al. (Duke University) developed novel compounds to improve Wnt/β-catenin selectivity. The structure of a selective compound evaluated in this study, SRI33576, was first identified by Mook et al. to have specificity for Wnt/β-catenin signaling over ATP homeostasis in colorectal cancer cells [40]. SRI32529 was first developed in a screen of thirty 6-nitrobenzimidazoles for phosphodiesterase inhibitors. SRI32529 was not selected as one

of the effective inhibitors [51]. In addition, SRI32529 was found to have less potency in inhibiting Wnt/β-catenin signaling than SRI33576 [40]. Neither compound has been evaluated for antiproliferative effects in cancer cells. Also, the effects of SRI33576 have not been distinguished from those of niclosamide on non-Wnt/β-catenin signaling pathways, such as mTOR, STAT3, and Notch. Benzimidazole compounds developed at Southern Research (SR) were screened for effects on cell viability and apoptosis in TNBC and selectivity for Wnt/β-catenin signaling. SRI33576 and SRI35889 were discovered to have more cytotoxic efficacy and specificity against Wnt/β-catenin signaling in TNBC than in noncancerous cells.

In the current study, modification of niclosamide has led to the identification of novel benzimidazole compounds that inhibit Wnt/β-catenin signaling. SRI33576 and SRI35889 are effective and selective Wnt/β-catenin inhibitors which do not affect mTOR, STAT3, and Notch signaling. We propose these compounds are potentially effective therapeutic agents for cancers dependent on inhibition of Wnt/β-catenin signaling.

RESULTS

SRI33576 and SRI35889 are more cytotoxic to TNBC than to noncancerous MCF10A cells

Five of the 2,5-disubstituted phenyl benzimidazoles were tested for cytotoxicity and inhibition of Wnt/β-catenin signaling (Table 1). First, it was important to compare efficacies of the SR compounds in inhibiting proliferation of TNBC and noncancerous cells. Both SRI33576 and SRI35889 exhibit more cytotoxicity in TNBC cell lines than in noncancerous MCF10A cells. IC50 values for SUM149, SUM159, MDA-MB-231, and

MDA-MB-468 TNBC cell lines treated with SRI33576 ranged from 1.9 to 3.2 μM, compared to 5.0 μM for MCF10A cells. IC50 values for TNBC cell lines treated with SRI35889 ranged from 1.1 μM to 2.4 μM, compared to 5.4 μM for treated MCF10A cells (Fig. 1). IC50 values for TNBC cell lines treated with niclosamide, an inhibitor of multiple signaling pathways, ranged from 0.3 μM to 1.0 μM, while the IC50 value for MCF10A cells was 1.0 μM. Thus, compared to SR agents, niclosamide exhibits less selectivity in eliciting cytotoxicity in TNBC cells vs. noncancerous cells.

SRI33576 and SRI35889 induce apoptosis in TNBC

Apoptosis of TNBC cell lines was evaluated by Annexin V/PI assay following treatment with the agents. Compounds SRI33576 and SRI35889 induced significantly more apoptosis in MDA-MB-231 and MDA-MB-468 cells treated with 5 μM for 48 hours. SRI35361 was the only compound which did not induce significantly more apoptosis than DMSO in either cell line (Figs. 2A and B). In addition, a higher percentage of MDA-MB-231 cells treated with 5 μM SRI33576 (24% of total) and SRI35889 (26.5%) resided in the late apoptotic phase as compared to vehicle control-treated cells (8.1%). This was comparable to cells treated with niclosamide (29.5%) as shown in Fig. 2C.

SRI33576 and SRI35889 inhibit Wnt/β-catenin signaling in TNBC cell lines but not noncancerous cells

We sought to determine specificity of the two compounds for inhibition of Wnt/β-catenin signaling in TNBC over noncancerous cells. As niclosamide is an inhibitor of

several pathways, we compared the effects of this agent with the SR benzimidazoles. Modulation of Wnt/β-catenin pathway-related proteins by the compounds was detected by Western blotting. Nuclear β-catenin promotes transcription of pro-tumorigenesis genes, and cytoplasmic β-catenin is associated with poor prognosis in breast cancer patients [52]. To detect cytoplasmic/nuclear active (non-phosphorylated) β-catenin, we used a monoclonal antibody which recognizes non-phosphorylated sites Ser-37 and Thr-41. The antibody has been shown to visualize production of active β-catenin via the canonical Wnt/β-catenin pathway during murine embryogenesis [53]. Interestingly, active β-catenin was observed in the cytoplasm of MDA-MB-468 cells but not MDA-MB-231 cells. SRI35889 decreased cytoplasmic β-catenin (Fig. 3A). SRI33576 and SRI35889 decreased nuclear active β-catenin expression following 18 h treatment (Fig. 3B). Immunocytochemistry also revealed decrease in nuclear active β-catenin expression with SRI33576 and SRISRI35889 treatment in MDA-MB-231 and MDA-MB-468 cells (Fig. 4).

Because the receptor LRP6 is an initiator of Wnt/β-catenin signaling, we sought to assess whether the compounds inhibited expression of the phosphorylated (active) form. SRI33576 decreased phosphorylated LRP6 at 5 μM in both TNBC cell lines but not MCF10A cells. SRI35889 decreased phosphorylated LRP6 at 2.5 and 5 μM in both TNBC cell lines but not MCF10A cells. Niclosamide inhibited decreased phosphorylated LRP6 in MCF10A cells (Fig.5).

SRI33576 and SRI35889 have less effects on mTOR, STAT3, and Notch signaling than niclosamide and less effects in TNBC than in MCF10A cells

Niclosamide affects mTOR, STAT3, and Notch pathways, potentially conferring increased toxicity in normal tissues. Specificity of benzimidazole compounds was evaluated by assessing their effects on non-Wnt/β-catenin signaling pathways mTOR, STAT3, and Notch. Cell lines were treated with the compounds and assayed by Western blotting for changes in protein levels associated with these pathways. Niclosamide downregulated STAT3 phosphorylation in TNBC and MCF10A cells, whereas SRI33576 and SRI35889 produced noticeably less of an effect (Fig. 5). SRI33576 inhibited phosphorylation of 4EBP1 at 5 μM in both TNBC cell lines, but it did not yield such a change in MCF10A cells. SRI35889 inhibited 4EBP1 phosphorylation at 2.5 and 5 μM in both TNBC cell lines, but it produced less of a change in MCF10A cells at 2.5 μM. Neither of the compounds downregulated phosphorylation of 4EBP1 as much as niclosamide (Fig. 5). Expression of the pSTAT3β and total STAT3β isoform (lower band), associated with tumor-suppressor function [54], was markedly decreased by niclosamide but not by SRI33576 or SRI35889 in MDA-MB-468 and MCF10A cells. SRI33576 and SRI35889 decreased expression of Wnt/β-catenin pathway proteins cyclin D1 and survivin in TNBC cells at 2.5 and 5 μM concentrations. At 2.5 μM concentration, SRI35889 did not affect cyclin D1 or survivin expression as much as niclosamide in MCF10A cells (Fig. 5).

DISCUSSION

Despite the fact Wnt/β-catenin signaling is not driven by mutations encoding pathway-related proteins, Wnt/β-catenin signaling represents an appealing target for

inhibitors due to higher activity in TNBC than in normal tissues. Niclosamide has been established as an effective inhibitor of Wnt/β-catenin signaling in several cancers, including colon, breast, and myeloma [36, 55-58]. Studies indicated niclosamide exhibits no carcinogenic effects in animals [38]. However, several studies have shown the agent elicits genotoxic effects [59-61]. SRI33576 was previously found to inhibit Wnt/β-catenin signaling in colorectal cancer cells by decreasing cytosolic axin2 and β-catenin and overall c-myc, survivin, and cyclin D1 levels [40]. However, it was not known whether nuclear β-catenin, the major driver of canonical Wnt/β-catenin signaling, was abrogated by the agent in colorectal cancer cells. To our knowledge, this is the first study evaluating the effects of 2,5-disubstituted phenyl benzimidazoles on TNBC cell lines.

Benzimidazoles SRI33576 and SRI35889 were identified with improved specificity for Wnt/β-catenin signaling in TNBC cell lines than in noncancerous cells. Compared to niclosamide, both agents preferentially inhibit Wnt/β-catenin signaling, indicated by lower expression of Wnt/β-catenin pathway proteins survivin and cyclin D1. A member of the inhibitor of apoptosis (IAP) family, survivin controls cell division, mitigates apoptosis, and suppresses active caspases [62-66]. Overexpression of cyclin D1, a driver of G1/S transition during cell cycle progression, has been observed in more than half of breast cancers [67]. Inhibition of Wnt/β-catenin signaling in TNBC is associated with promotion of apoptosis [5]. Both agents increased apoptotic levels in TNBC cells, potentially due to their effects on Wnt/β-catenin signaling. In addition, it appears that compounds SRI33576 and SRI35889 decrease active β-catenin expression via targeting of LRP6 in TNBC cells over noncancerous cells. As TNBC is characterized by high LRP6 expression [16, 20], targeting of this receptor by the compounds

underscores their therapeutic relevancy. In addition, the inhibitors exhibited less pronounced effects on mTOR signaling in TNBC cell lines than in noncancerous cells. Neither SRI33576 nor SRI35889 affected Notch or STAT3 signaling activities in TNBC cell lines.

It is known autocrine Wnt/β-catenin signaling drives TNBC cell proliferation [17]. Both SRI33576 and SRI35889 inhibited autocrine Wnt/β-catenin signaling using established human cancer cell lines. However, paracrine signaling via activity of ligands such as Wnt3a may also play a role in TNBC growth [19], and it remains to be elucidated whether SRI33576 and SRI35889 can inhibit this pathway.

We previously evaluated other novel analogs of niclosamide that inhibited Wnt/β-catenin and mTOR signaling [68, 69]. SRI35889, and to a lesser extent, SRI33576, were found to inhibit mTOR signaling in addition to Wnt/β-catenin activity, which may confer increased cytotoxic potency in TNBC. Recently, a study supported the use of dual inhibition of both PI3K/AKT/mTOR and Wnt/β-catenin pathways for synergistic effects in TNBC [70]. Interestingly, treatment of TNBC with the pan-PI3K inhibitor buparlisib increased Wnt/β-catenin activity via increased expression of Wnt/β-catenin pathway mediators such as FZDs, Wnt ligands, LRP4/6, and porcupine. Unfortunately, the inhibitor was found to elicit toxic effects, such as anxiety, pneumonititis, and liver toxicity, in humans.

An appealing target of new therapies, CSCs participate in tumor recurrence, metastasis, and resistance to chemotherapies in breast cancer. Evidence indicates β-catenin expression correlates with chemoresistance of TNBC as β-catenin knockdown sensitized TNBC cell lines to doxorubicin- or cisplatin-mediated cell death [13]. Wnt/β-

catenin pathway inhibitors niclosamide, its analogs, and salinomycin were found to effectively target breast and ovarian CSCs [47, 69, 71-75]. Because Wnt/β-catenin signaling mediates self-renewal and migration of CSCs, SRI33576 and SRI35889 may represent clinically useful therapeutic agents.

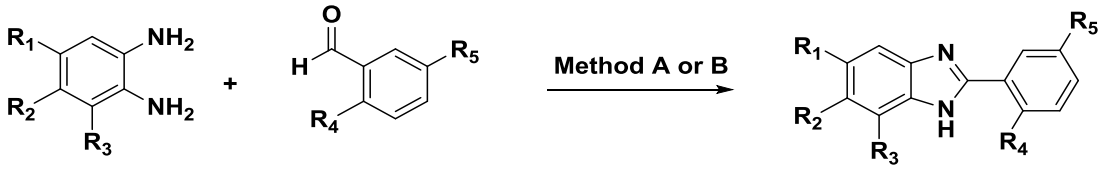
This study supports SRI33576 and SRI35889 for further investigation in animal studies. These compounds must be further evaluated for their PK/PD properties to elucidate their potential as cancer-targeting agents. In addition, both compounds may be combined with chemotherapy or immunotherapeutic agents to further enhance their therapeutic effects.

MATERIALS AND METHODS

Compound synthesis

4.1. Compound synthesis

Synthesis of compounds SRI32529 and SRI33576 have been previously described [40, 51].



Method A: NaHSO3, DMSO, 210oC, 1h; Method B: Na2S2O5. DMF, 170oC, 15 min, MW

Method A utilized the condensation of diamines and aldehydes in presence of sodium sulfite and DMSO at 210oC for 1 hour. An improved synthetic method (Method B) was determined which utilized microwave conditions in the reaction of the substituted

diamines and aryl rings in the presence of sodium metabisulfite and dry DMF. Method B was preferred versus Method A due to shorter reaction times (15 min) and higher yields.

Method A. A mixture of an appropriate 1,2-phenylenediamine (1.0 eq), appropriate aldehyde (1.0 eq), and sodium bisulfite (1.0 eq) in 10 mL of DMSO was heated at 210oC for 1 h. The reaction mixture was filtered, concentrated in vacuo and purified on a purification system using 10-50% hexanes and EtOAc.

Method B. A mixture of an appropriate 1,2-phenylenediamine (1.0 eq), appropriate aldehyde (1.0 eq), and sodium metabisulfite (1.0 eq) in 8 mL of DMF was heated in a CEM microwave at 170oC for 15 min. The reaction mixture was filtered, concentrated in vacuo and purified on a purification system using 10-50% hexanes and EtOAc.

2-(5,7-Dichloro-3H-benzo[d]imidazol-2-yl)-4-fluorophenol (35357). This compound was prepared from 3,5-dichlorobenzene-1,2-diamine and 5-fluoro-2-hydroxybenzaldehyde using Method A. Yield 21%. TLC Rf = 0.40 (Hexanes–EtOAc, 2:1). 1H NMR (400 MHz, DMSO-d6) δ 13.40 (s, 1H), 7.95 (s, 1H), 7.71 (s, 1H), 7.48 (s, 2H), 7.30 (ddd, J = 9.2, 8.1, 3.1 Hz, 1H), 7.09 (dd, J = 9.1, 4.7 Hz, 1H). HRMS m/z calcd for C13H7Cl2FN2O [M+H]+: 296.9992, found: 296.9993.

2-(5-Chloro-6-fluoro-1H-benzo[d]imidazol-2-yl)-4-fluorophenol (35361). This compound was prepared from 4-chloro-5-fluorobenzene-1,2-diamine and 5-fluoro-2-hydroxybenzaldehyde using Method A. Yield 64%. TLC Rf = 0.45 (Hexanes–EtOAc, 2:1). 1H NMR (400 MHz, DMSO-d6) δ 12.60 (s, 1H), 8.54 (s, 1H), 8.22 – 8.11 (m, 2H), 7.82 (d, J = 8.9 Hz, 1H), 7.24 – 7.09 (m, 1H). HRMS m/z calcd for C13H7ClF2N2O [M+H]+: 280.0215, found: 280.0215.

4-Chloro-2-(5,6-dichloro-1H-benzo[d]imidazol-2-yl)phenol (35889). This compound was prepared from 4,5-dichlorobenzene-1,2-diamine and 5-chloro-2-hydroxybenzaldehyde using Method B. Yield 92%. TLC Rf = 0.30 (Hexanes–EtOAc, 1:1). 1H NMR (400 MHz, DMSO-d6) δ 12.99 (s, 1H), 8.24 (s, 1H), 7.95 (s, 1H), 7.70 (q, J = 1.8 Hz, 1H), 7.45 - 7.40 (m, 2H), 7.08 (dd, J = 8.9, 2.4 Hz, 1H). HRMS m/z calcd for C13H7Cl3N2O [M+H]+: 312.9697, found: 312.9696. HPLC: 100% (tR = 3.05 min).

Materials

All experimental compounds were synthesized at Southern Research (Birmingham, AL) and dissolved in DMSO at stock concentrations of 10 mM. Antibodies purchased from Cell Signaling Technologies (Danvers, MA) include phospho-LRP6 (#2568), LRP6 (#2560), cyclin D1 (#2978), survivin (#2808), phospho-4EBP1 (#9451), 4EBP1 (#9452), phospho-STAT3 (Tyr705) (#9145), STAT3 (#4904), Hes1 (#11988), α-tubulin (#2144), and lamin A/C (#2032). Tissue culture media were obtained from ThermoFisher (Waltham, MA) and FBS was obtained from Atlanta Biologicals (Flowery Branch, GA). Niclosamide, insulin, cholera toxin, hydrocortisone, protease and phosphatase inhibitor cocktails were purchased from Sigma-Aldrich (St. Louis, MO). Epidermal growth factor was obtained from PeproTech (Rocky Hill, NJ).

Cell culture

Nonmalignant mammary cell line MCF10A and TNBC cell lines MDA-MB-231 and MDA-MB-468 were purchased from ATCC (Manassas, VA). MCF10A cells were cultured in DMEM/F-12 (1:1) supplemented with 5% horse serum, 10 µg/mL insulin, 0.5

µg/mL hydrocortisone, 0.02 µg/mL epidermal growth factor, 0.1 µg/mL cholera toxin. MDA-MB-231 and MDA-MB-468 cell lines were cultured in DMEM with 10% FBS. SUM149 and SUM159 cell lines were purchased from Asterand (Detroit, MI) and cultured in DMEM/F12 with 5% FBS, hydrocortisone, and insulin. All cell lines were maintained in an incubator at 37ºC and 5% CO2.

In vitro cytotoxicity

MCF10A and TNBC cells (2 × 103) were seeded with appropriate growth media containing 10% FBS in each well of tissue culture-treated 96-well black plates (Corning #3904)(Corning, NY) 16 h prior to treatment. Cells were treated with SR compounds for 3 days at concentrations of 0-5 µM. Cell viability was then evaluated with Cell Titer Glo (Promega, Madison, WI), and luminescence was measured using a TOPCount NXT plate reader (PerkinElmer, Waltham, MA). Curves were fit according to nonlinear regression and IC50 values were calculated using GraphPad Prism 7. Cells were treated in quadruplicates and experiments were performed three times.

Apoptosis assay

Cells were seeded in 6-well tissue-culture treated plates (Corning) for overnight incubation. Culture media was replaced with growth media containing 1% FBS. Cells were treated with compounds for 48 h prior to harvest. Apoptosis/necrosis was detected by the FITC Annexin V Apoptosis Detection Kit I from Becton Dickinson (San Jose, CA). Cells positive for Annexin V, propidium iodide (PI) or both were all designated as apoptotic.

Western blot analysis

Cells were seeded in 6-well tissue-culture treated plates for 48 h until 80% confluency prior to treatment. Whole cell extracts were prepared with Laemmli buffer (5% SDS, 10% glycerol, 0.5 M Tris-Cl) supplemented with protease and phosphatase inhibitors, and equivalent amounts of proteins were separated by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA) and blocked with 5% BSA in tris-buffered saline (TBS) supplemented with 0.1% Tween-20 for 1 h. Membranes were incubated with primary antibody overnight on a shaker at 4ºC. They were then washed and incubated with secondary antibody for 1 h. Bands were visualized with the Enhanced Chemiluminescence Detection reagents (PerkinElmer).

Cells were lysed using the CERI and CERII reagents of the cytoplasmic-nuclear extraction kit (ThermoFisher,) to obtain cytoplasmic fractions following treatment. Nuclear fractions were isolated by using 1X Laemmli buffer and sonication for a few seconds. Membrane was incubated overnight with active β-catenin (MilliporeSigma, Burlington, MA), lamin A/C, and tubulin antibodies.

Immunocytochemistry

Cells were seeded on coverslips for 48 h prior to treatment with DMSO and SR compounds for 18 h. Cells were washed with PBS, fixed with 4% paraformaldehyde for 20 minutes, and permeabilized with 0.5% Triton X-100 for 20 minutes. Following blocking with 3% BSA for 30 minutes, cells were incubated overnight with active β-

catenin antibody (Millipore) at 4ºC. Cells were incubated with Alexa 488 goat anti-mouse secondary antibody (1:1000) at room temperature for 1 h. To counterstain, cells were incubated with 1:5000 Hoechst 33342 for 20 minutes. Coverslips were mounted with Prolong Gold Antifade Reagent (ThermoFisher), and cells were observed with Nikon A1R confocal microscope (Nikon Instruments, Melville, NY). Images were obtained at 20X magnification.

Statistical analysis

Statistical analyses were performed by the unpaired Student t test. $P < 0.05$ values were considered significantly different. Results are shown as mean ± SD.

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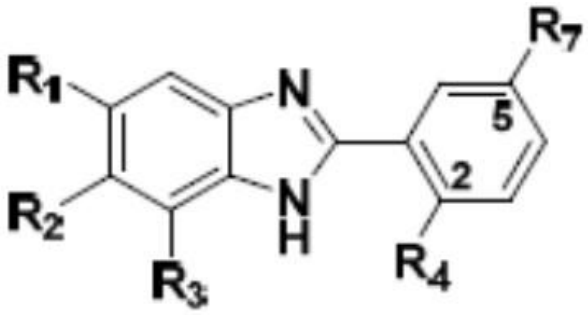


	R_1	R_2	R_3	R_4	R_7
SRI32529	H	NO_2	H	OH	Cl
SRI33576	H	CF_3	H	OH	Cl
SRI35357	Cl	H	Cl	OH	F
SRI35361	F	Cl	H	OH	Cl
SRI35889	Cl	Cl	H	OH	Cl

Table 1. Scaffold and structures of 2,5-disubstituted phenyl benzimidazoles. Compounds SRI32529, SRI33576, SRI35357, SRI35361 and SRI35889 were evaluated for cytotoxicity and inhibition of Wnt/β-catenin signaling.

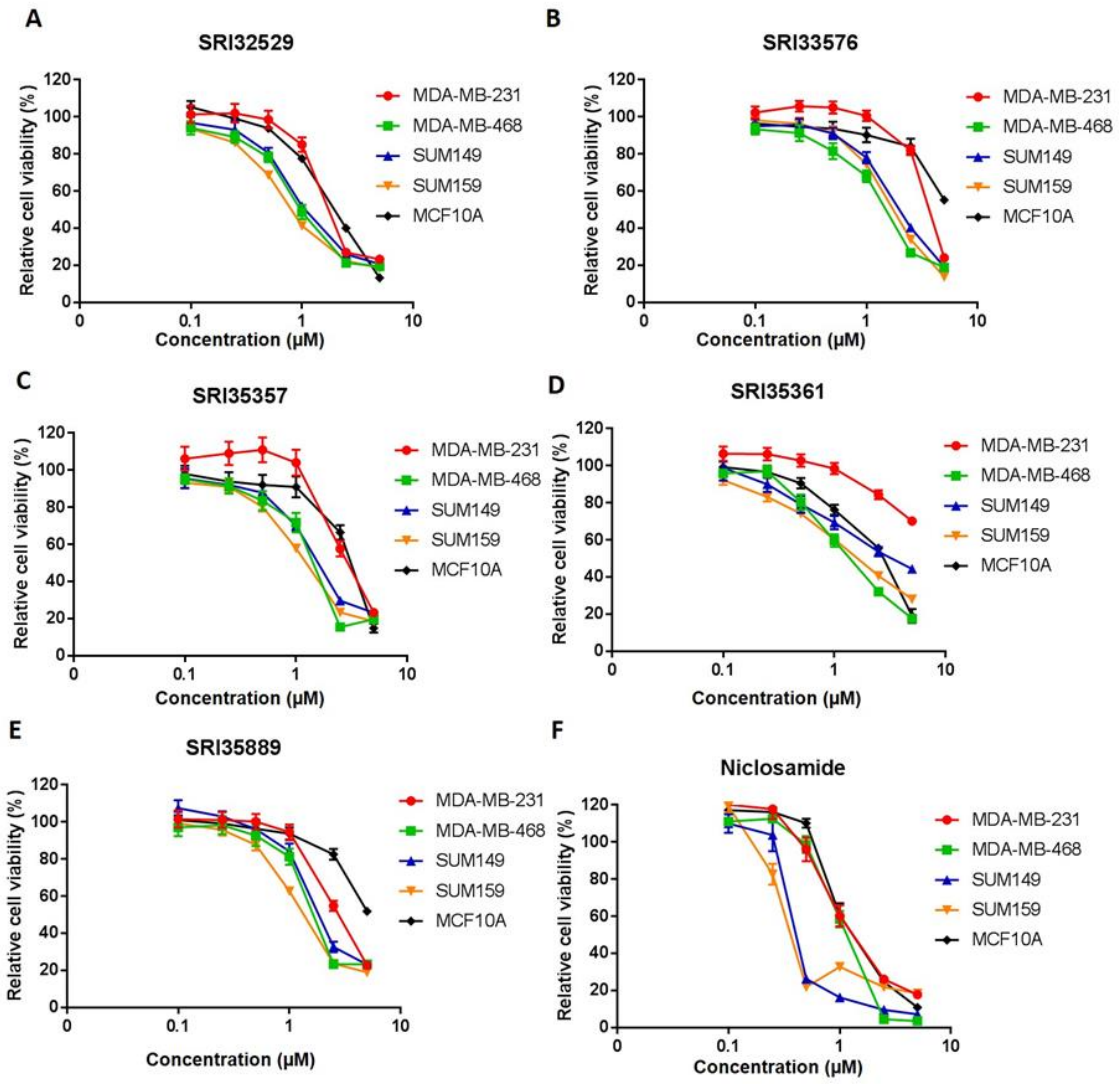
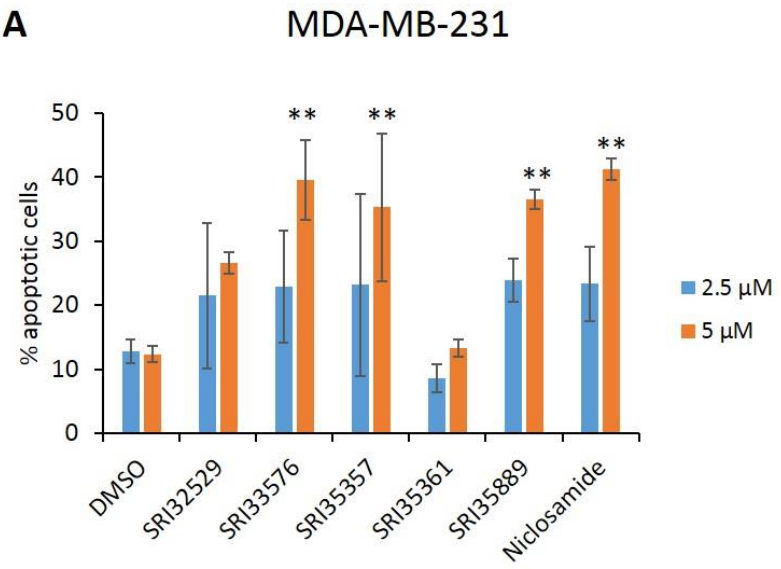
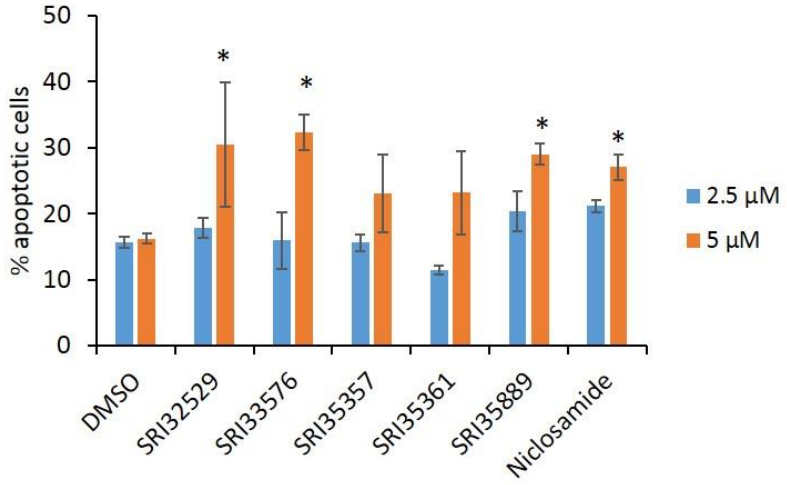


Figure 1. Effects of benzimidazole compounds on viability of TNBC and noncancerous MCF10A cells. Cells were treated with 0-5 μM SRI32529 (A), SRI33576 (B), SRI35357 (C), SRI35361 (D), SRI35889 (E), and niclosamide (F) for 72 h. Assays were performed three times.

A


MDA-MB-231
% apoptotic cells
50
40
30
20
10
0
**
**
**
**
DMSO
SRI32529
SRI33576
SRI35357
SRI35361
SRI35889
Niclosamide
2.5 µM
5 µM

B


MDA-MB-468
% apoptotic cells
50
40
30
20
10
0
*
*
*
*
DMSO
SRI32529
SRI33576
SRI35357
SRI35361
SRI35889
Niclosamide
2.5 µM
5 µM

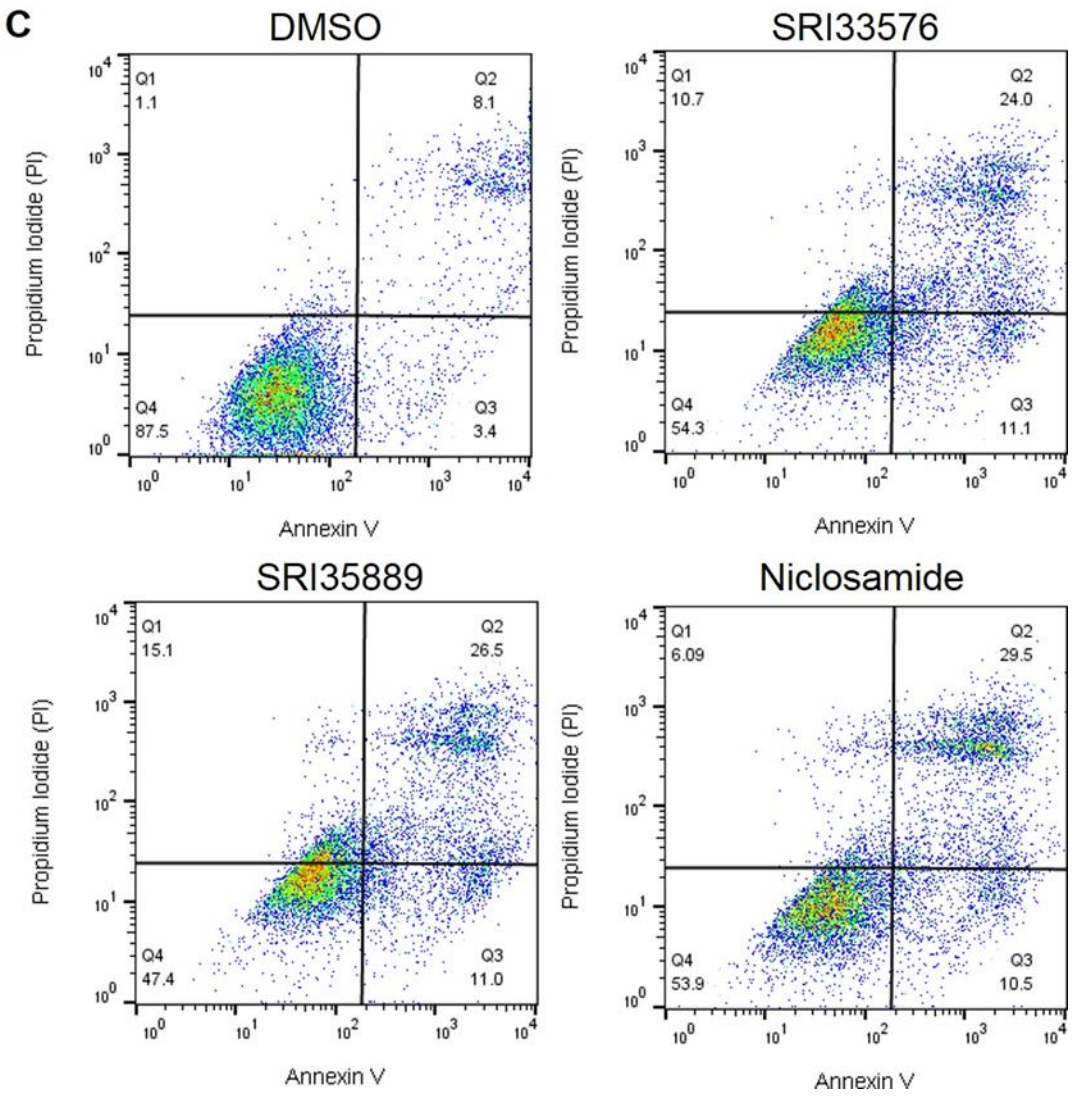


Figure 2. Apoptosis of (A) MDA-MB-231 and (B) MDA-MB-468 TNBC cells following treatment with SR compounds and niclosamide for 48 h. Apoptosis was measured by staining cells with Annexin V and PI prior to analysis by flow cytometry. Apoptosis of cells treated with compounds was compared to DMSO-treated cells. (C) Representative scatterplots of MDA-MB-231 cells treated with DMSO, 5 μM SRI33576, SRI35889, and niclosamide for 48 h. Assays were performed three times. *P < 0.05, **P < 0.01.

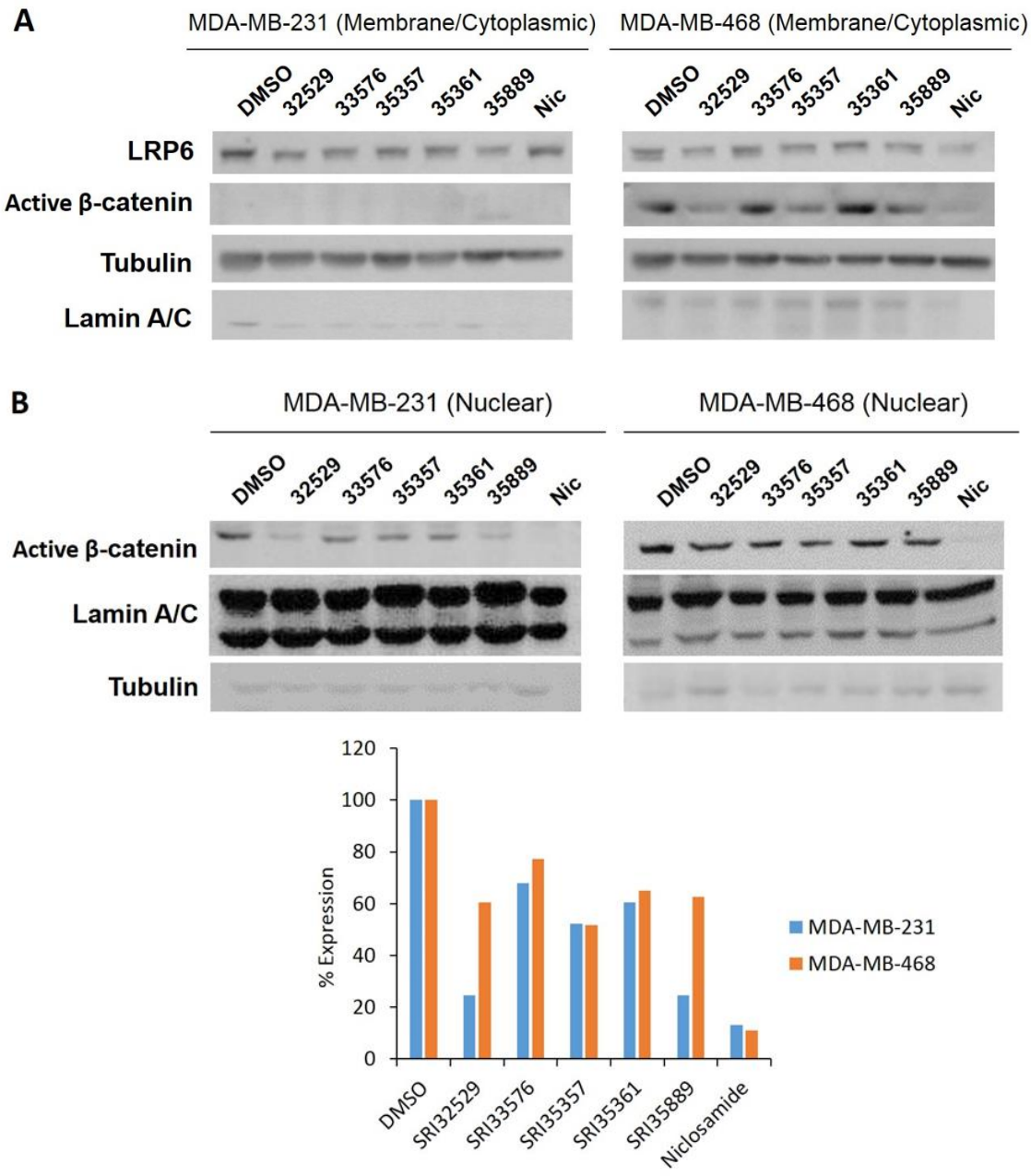
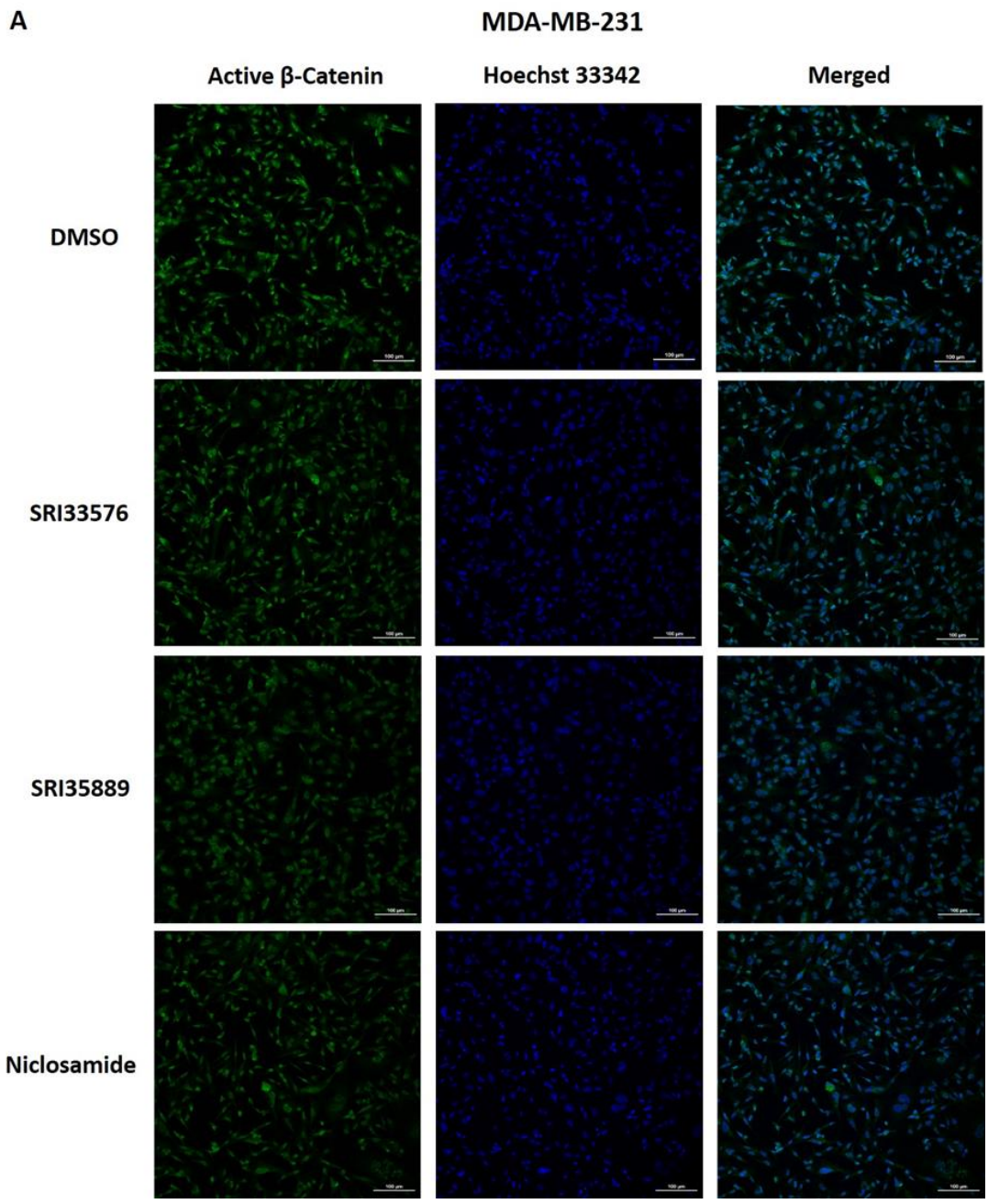
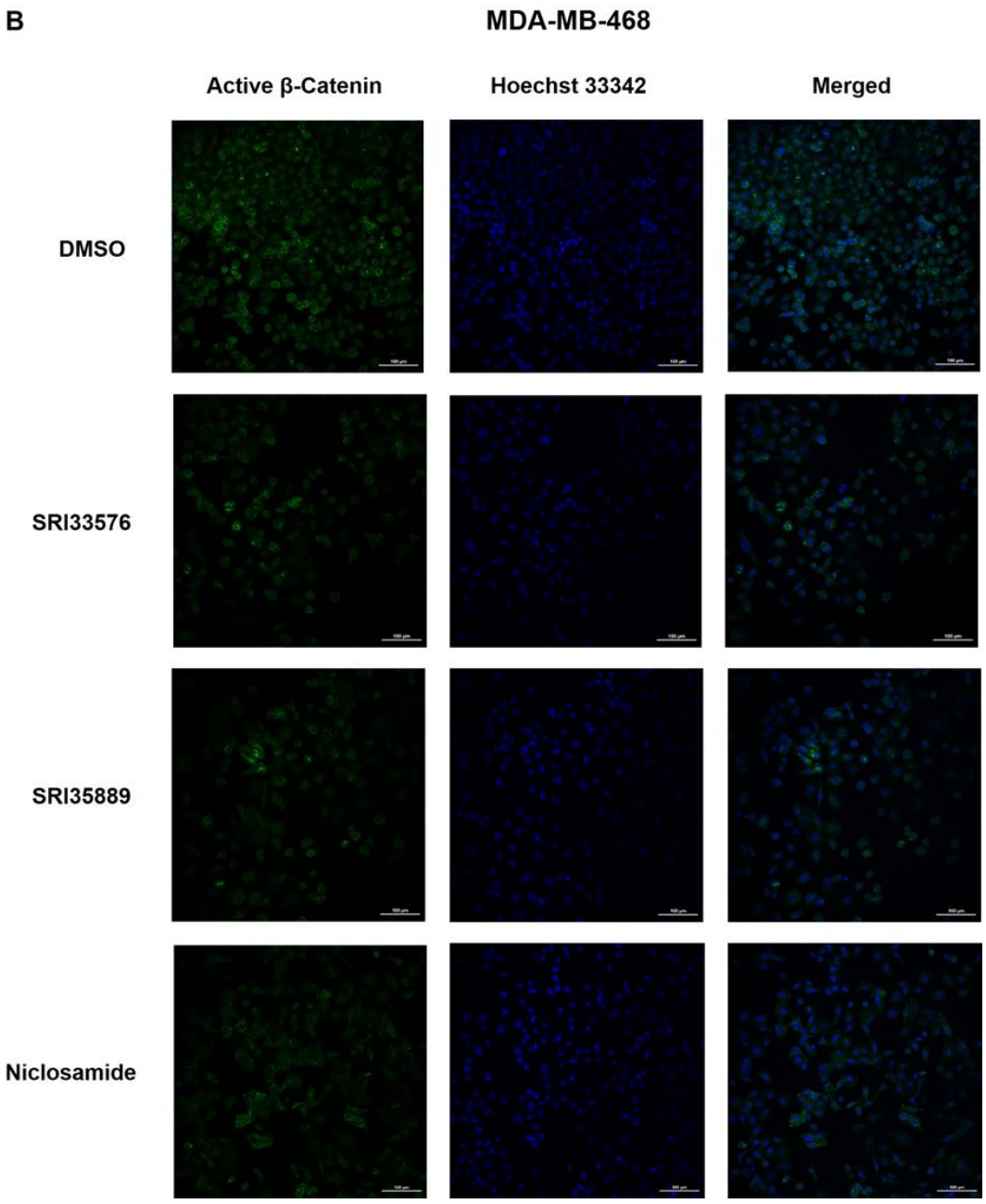


Figure 3. Effects of SR compounds and niclosamide on active β-catenin expression in TNBC cells. (A) Cytoplasmic and (B) nuclear active β-catenin expression was evaluated in the cells following 18 h treatment with 5 μM concentrations of the compounds and cytoplasmic-nuclear fractionation. Intensity of nuclear active β-catenin bands in (B) were quantified by densitometry with ImageJ software and normalized to corresponding band of lamin A/C. Western blotting was performed on lysates (40 ug loaded). Active β-catenin was not detected in cytoplasm of MDA-MB-231 cells. Tubulin and lamin A/C were detected to ensure proper separation of cytoplasmic and nuclear fractions. Western blotting was performed two times.


A
MDA-MB-231
Active β-Catenin
Hoechst 33342
Merged
DMSO
SRI33576
SRI35889
Niclosamide


B
MDA-MB-468
Active β-Catenin
Hoechst 33342
Merged
DMSO
SRI33576
SRI35889
Niclosamide

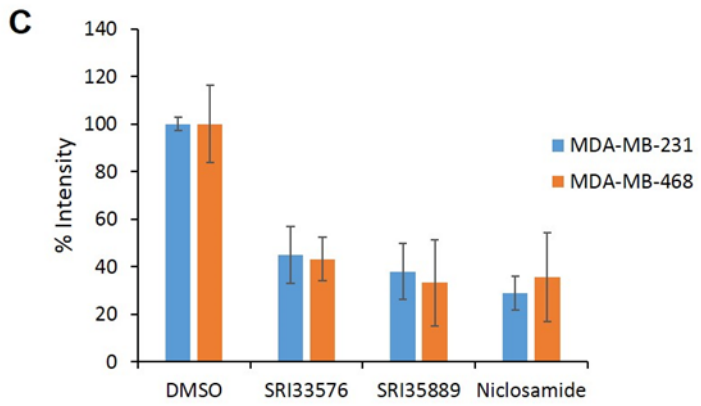
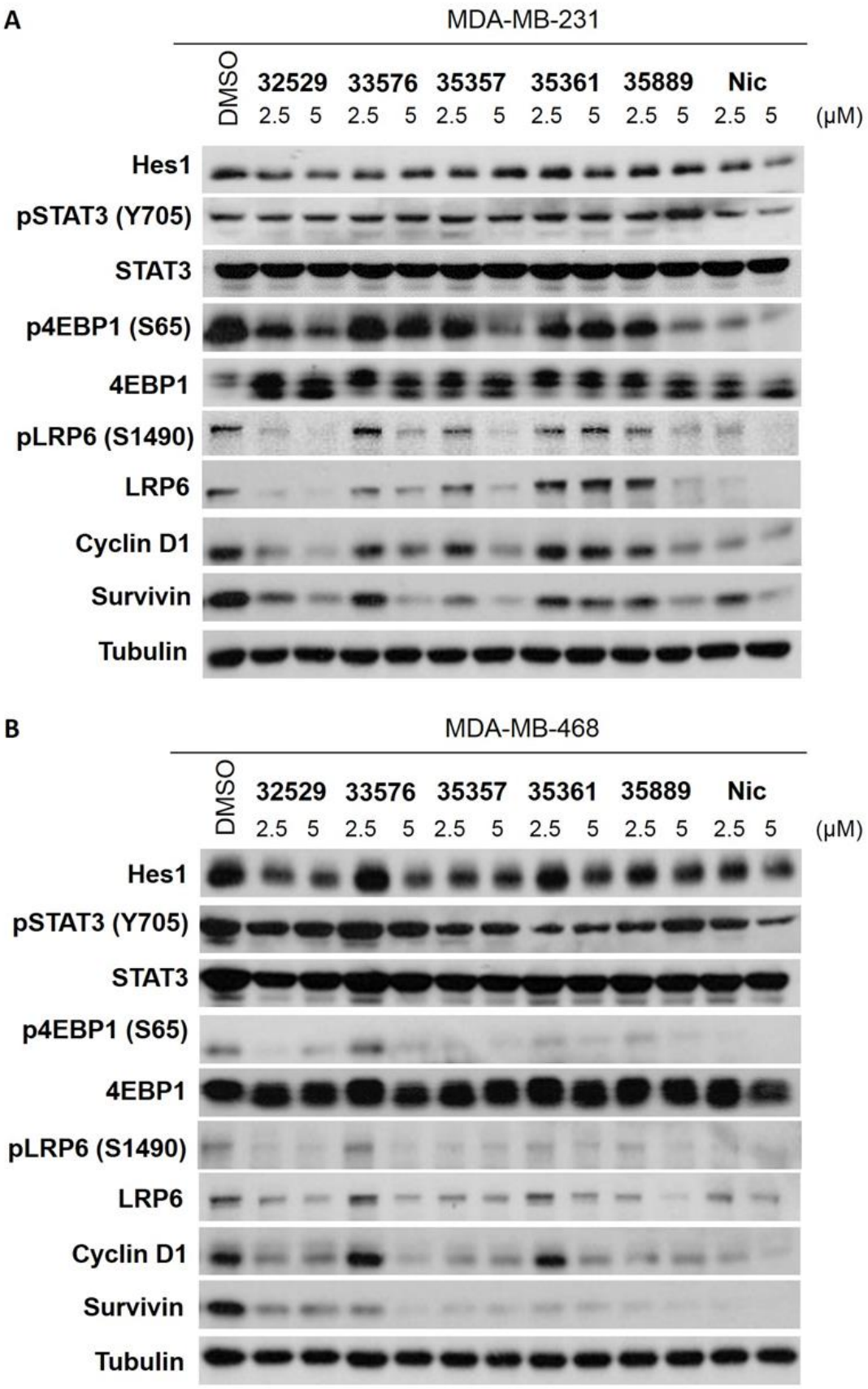


Figure 4. Immunocytochemistry (ICC) of active β-catenin following treatment with SR compounds. IF of active β-catenin in (A) MDA-MB-231 and (B) MDA-MB-468 cells treated with 0.05% DMSO, 5 µM SRI33576, SRI35889, and niclosamide. Cells were counterstained with Hoechst 33342 (blue). (C) Fluorescence intensities were quantified by ImageJ, and means were compared to DMSO-treated cells (n = 3).


A
MDA-MB-231
DMSO
32529
33576
35357
35361
35889
Nic
2.5 5 2.5 5 2.5 5 2.5 5 2.5 5 2.5 5 (μM)
Hes1
pSTAT3 (Y705)
STAT3
p4EBP1 (S65)
4EBP1
pLRP6 (S1490)
LRP6
Cyclin D1
Survivin
Tubulin
B
MDA-MB-468
DMSO
32529
33576
35357
35361
35889
Nic
2.5 5 2.5 5 2.5 5 2.5 5 2.5 5 2.5 5 (μM)
Hes1
pSTAT3 (Y705)
STAT3
p4EBP1 (S65)
4EBP1
pLRP6 (S1490)
LRP6
Cyclin D1
Survivin
Tubulin

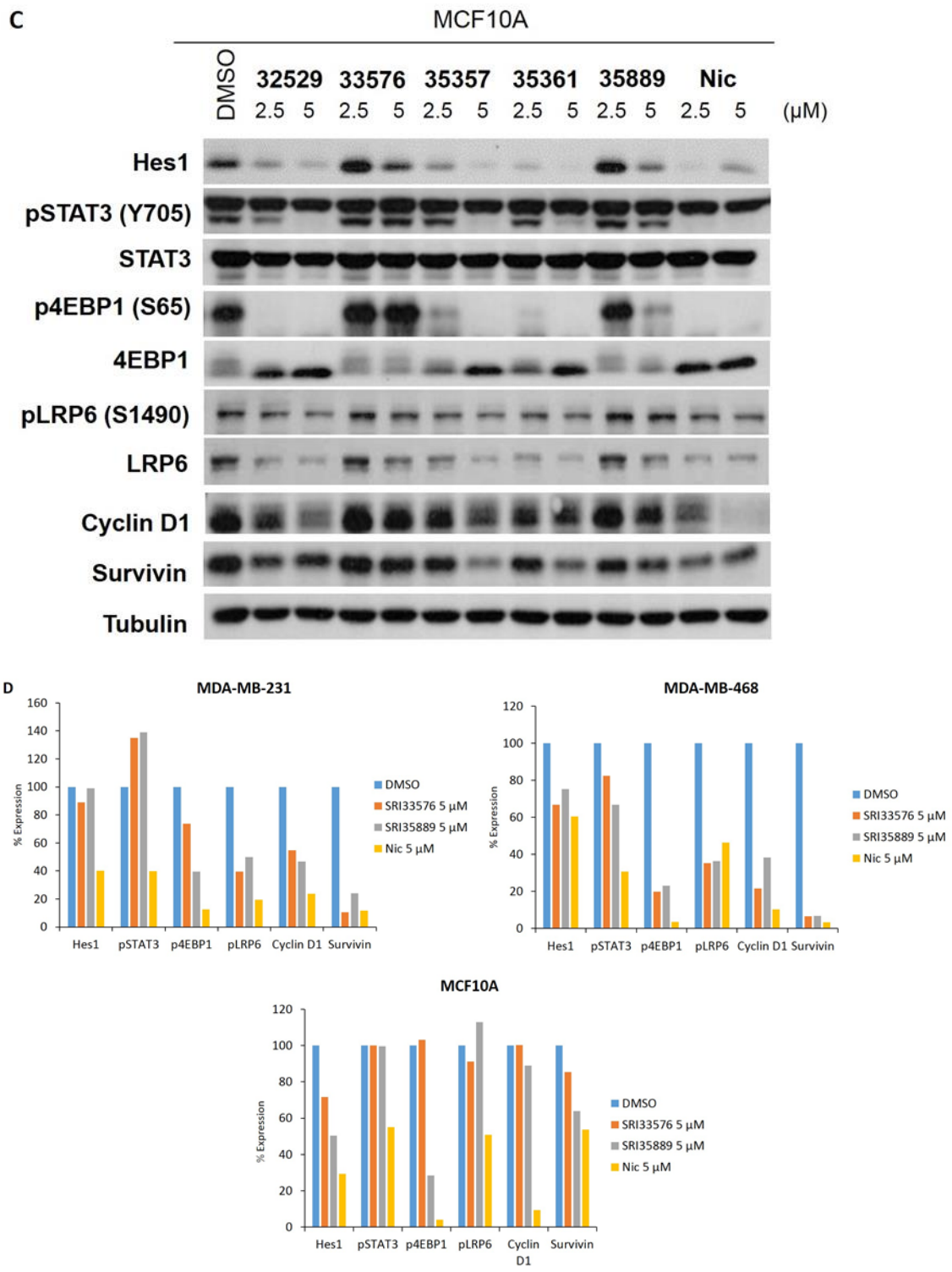


Figure 5. Effects of SR compounds on Wnt/β-catenin signaling in (A) MDA-MB-231, (B) MDA-MB-468, and (C) MCF10A cells treated with 2.5 or 5 μM of compounds for 18 h. Hes1, pSTAT3 (Y705), STAT3, p4EBP1 (p65), 4EBP1, pLRP6, cyclin D1, survivin, and tubulin in whole cell lysates were detected by immunoblotting. (D)

Densitometry readings for select proteins Hes1, pSTAT3, p4EBP1, pLRP6, LRP6, cyclin D1, and survivin according to western blots. Results are averages of two replicate experiments and normalized to tubulin. 0.05% DMSO was used as vehicle control. Western blotting was performed two times.

GENERAL DISCUSSION AND FUTURE DIRECTIONS

Because Wnt/β-catenin signaling is implicated in cancer development, progression, and metastasis, the pathway represents an appealing target for therapeutic agents. Our laboratory had previously evaluated efficacy of niclosamide against basal-like breast cancer pleural effusions [72] and ovarian cancer patient ascites samples [73]. While the agent displayed potency in *in vitro* studies, it did not show comparable effects *in vivo* due to its limited bioavailability and activity against multiple pathways. The agent's lack of bioavailability due to minimal absorption from the intestinal tract and poor water solubility necessitate development of alternative compounds. Off-target effects of niclosamide may also contribute to normal tissue toxicity and minimal efficacy in *in vivo* tumor models. This study examined effectiveness of novel compounds against Wnt/β-catenin signaling in ovarian and TNBC cell lines. We evaluated the effectiveness of novel niclosamide analogs in inhibiting ovarian bulk and CSC proliferation and the capacity to inhibit Wnt/β-catenin activity. In addition, we investigated cytotoxicity and selectivity of novel benzimidazole compounds against Wnt/β-catenin signaling in TNBC.

WNT974 Conclusions

WNT974 inhibits porcupine activity, modification of Wnt ligands prior to secretion, and Wnt/β-catenin signaling. Attenuation of Wnt secretion mitigates LRP6

activation and downstream Wnt/β-catenin signaling activity. WNT974 decreased phosphorylated LRP6, β-catenin, and Wnt targets cyclin D1 and survivin in a patient ascites sample (Figure 1). Following isolation of ascites cells from 53 patients with serous ovarian cancer, we evaluated potential cytotoxicity or inhibition of proliferation by trypan blue staining. As cell viability was not affected by treatment, we concluded WNT974 was not cytotoxic. Flow cytometry experiments indicate cell cycle arrest confers inhibition of proliferation of ovarian cancer ascites cells. Expression of E-cadherin was similar in both untreated and treated ascites cells, indicating absence of EMT. Although it has been reported RSPO fusion transcripts are present in ovarian cancer, we did not identify such plentiful fusions in tumors or ascites. No association between RSPO3 gene expression levels and sensitivity to WNT974 could be identified. However, RSPO3 expression correlated with sensitivity to combinatory WNT974 and carboplatin treatment. In addition, RSPO3 gene expression was observed as higher in carboplatin-resistant cells, confirming prior investigations revealing increased Wnt/β-catenin signaling in platinum-resistant cells [101-103].

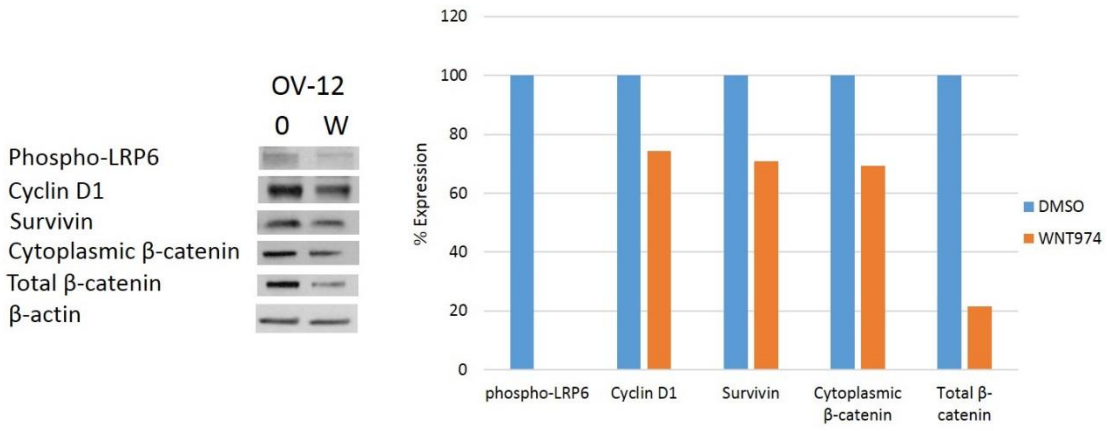


Figure 1. Effects of WNT974 on Wnt/β-catenin-related proteins in ascites cells. Patient ascites cells were treated with vehicle control DMSO (0) and 1 μM WNT974 (W) for 72 hours. Western blotting for lysates from the cells was performed to evaluate

expression of phospho-LRP6, cyclin D1, survivin, cytoplasmic and total β-catenin. Graph shows densitometric values normalized to β-actin as analyzed by ImageJ software (NIH).

Niclosamide Analogs Conclusions

We first decided to evaluate sensitivity of ovarian cancer cell lines to niclosamide analogs. Proliferation of ovarian cancer cell lines A2780 and SKOV3 and their chemoresistant counterparts, A2780cp20 and SKOV3Trip2, was inhibited by both analogs 11 and 32 to extents comparable to niclosamide. Because single-agent chemotherapy enriches for tumor cells with high Wnt/β-catenin signaling activity and proliferation, we sought to examine whether cytotoxicity was enhanced by combination treatment with chemotherapy. Inhibition of cell proliferation was significantly greater with the combination of either analog 11 or 32 with chemotherapy than with either agent alone. ATPlite assay was performed to evaluate cytotoxicity of the analogs. However, due to the possibility the analogs could affect ATP production via mitochondrial uncoupling rather than direct cytotoxic activity, cell cycle analysis was also performed. As the analogs were found to elicit cell cycle arrest in the G1 phase, ATP levels likely decreased due to cell cycle arrest rather than cytotoxic effects. Cells expressing CD133, a well-established marker for ovarian CSCs, were markedly reduced by treatment with niclosamide and analog 32. We next decided to evaluate the effects of the analogs on chemosensitive and chemoresistant cells derived from PDX mouse models. Sensitivities of the cells in tissue culture-treated or ultra-low attachment plates were similar between the analogs and niclosamide.

Next, we decided to evaluate capacity of analogs to inhibit Wnt/β-catenin signaling in ovarian cancer cell lines by Western blot analysis. Analogs 11 and 32

decreased expression of Wnt surface receptor LRP6, β-catenin, and Wnt-target proteins cyclin D1 and survivin in A2780ip2 and A2780cp20 cells comparable to niclosamide. Because mTOR signaling is implicated in ovarian cancer cell growth [104], we decided to evaluate whether the pathway was affected by the analogs. The analogs decreased protein levels of mTOR-related proteins 4E-BP1, phospho-4E-BP-1, p70S6K, and phospho-p70S6K to a similar extent as niclosamide in A2780ip2 and A2780cp20 cells.

To observe effects of the analogs on functional Wnt/β-catenin-induced transcription, we performed the TOPFlash assay. Interestingly, inhibition of β-catenin-mediated transcription was greater in A2780cp20 cells than A2780ip2 cells. In addition, greater inhibition of proliferation by niclosamide and analog 11 was observed in chemoresistant ovarian cancer PDX cells than the chemosensitive counterpart. This may be due to the fact chemoresistant cells display higher innate Wnt/β-catenin signaling, leading to greater sensitivity to inhibition.

Benzimidazole Compounds Conclusions

Our laboratory had previously evaluated efficacy of niclosamide in breast cancer stem cells and ovarian cancers. While the agent displayed potency in *in vitro* studies, it did not show comparable effects *in vivo* due to its limited bioavailability and activity against multiple pathways. Many inhibitors of Wnt/β-catenin signaling in cancer are limited by their interactions with other pathways, often leading to toxicity in normal tissues. Because the analogs were also found to affect other pathways such as mTOR and

STAT3, we decided to seek to identify novel compounds with greater targeting specificity for Wnt/β-catenin signaling and potential for treating TNBC *in vivo*.

2,5-disubstituted phenyl benzimidazole compounds had never been evaluated against TNBC cell lines. One compound, SRI33576, was previously identified as efficacious in inhibiting Wnt/β-catenin signaling in colorectal cancer cells [94]. However, the study did not investigate cytotoxic potential of the agent in breast cancer cells, its selectivity for Wnt/β-catenin signaling, or its effects in noncancerous cells. The study also only evaluated the effects of the compound on cytoplasmic β-catenin but not nuclear β-catenin, which is the driver of Wnt/β-catenin signaling. We selected SRI33576 and four other novel benzimidazole compounds for further evaluation of their cytotoxicity and selectivity against Wnt/β-catenin signaling. To identify compounds with selective cytotoxicity in TNBC over noncancerous cells, I treated TNBC and MCF10A cells with SR compounds and niclosamide. Compared to MCF10A cells, TNBC cell lines were far more sensitive to SRI33576 and SRI35889. In addition, niclosamide was cytotoxic against both TNBCs and noncancerous cells. Also, I decided to evaluate SRI33576 and SRI35889 for their ability to elicit apoptosis in TNBC cell lines. SRI33576 and SRI35889 were found to elicit more apoptosis in MDA-MB-231 and MDA-MB-468 cells than the other SR agents. The two compounds induced apoptosis in both cell lines at similar levels as niclosamide. Interestingly, following 48 hours of treatment, there was a large shift of MDA-MB-231 cells into the late apoptotic phase. MDA-MB-468 cells were predominantly shifted into the early apoptotic phase (data not shown).

It has been postulated that the property of niclosamide to target proliferation signaling pathways other than Wnt/β-catenin signaling confers limited therapeutic

effectiveness and toxic side effects in normal tissues. I examined the selectivity of the benzimidazole compounds against Wnt/β-catenin signaling by comparing their inhibitory effects on Wnt/β-catenin pathway mediators LRP6, active β-catenin, cyclin D1, and survivin with niclosamide in both TNBC cells and nonmalignant MCF10A cells. LRP6 drives Wnt/β-catenin signaling, cell proliferation, and *in vivo* tumor growth [34]. Li et al. reported that niclosamide promotes LRP6 degradation and abrogates Wnt/β-catenin signaling [76]. Both SRI33576 and SRI35889 decreased LRP6 levels, further supporting their effectiveness as Wnt/β-catenin signaling inhibitors.

Nuclear active β-catenin was nearly undetectable in MCF10A cells by immunofluorescence and Western blotting (results not shown), supporting prior evidence that Wnt/β-catenin signaling activity is higher in TNBC compared to nonmalignant cells. Because 5 μM SRI33576 did not decrease nuclear active β-catenin in MDA-MB-231 cells following 18 hours of treatment, we decided to prolong treatment for 24 hours to investigate a possible dose-dependent effect. Nuclear β-catenin decreased with SRI33576 and SRI35889 treatment at 5 μM concentration. Interestingly, SRI32529, SRI35357, and SRI35889 decreased nuclear β-catenin more profoundly than SRI33576 and SR35361. SRI32529, SRI35357, and SRI35889 were also more cytotoxic against MDA-MB-231 and MDA-MB-468 cells. The correlation between decreased nuclear β-catenin levels and sensitivity to the compounds underscores the reliance of TNBC cell proliferation on Wnt/β-catenin signaling and the importance of pathway inhibition in treatment.

To evaluate selectivity of compounds for Wnt/β-catenin signaling over other pathways, I compared their effects with niclosamide on mTOR, STAT3, and Notch

activities by Western blotting. Niclosamide affected mTOR, STAT3, and Notch signaling in TNBC and MCF10A cells to a greater degree than either SRI33576 or SRI35889. The capacity of niclosamide to affect multiple pathways may confer its enhanced cytotoxicity in TNBC and nonmalignant cells.

Future Directions

Canonical Wnt/β-catenin signaling is a complex pathway with many components and a lack of druggable targets. The exact target(s) of the two benzimidazole compounds SRI33576 and SRI35889 remains to be elucidated. Mechanisms of action by the agents can be valuable to understanding the reasoning behind their specificity and may prompt development of improved targeting agents against TNBC over normal tissues.

To elucidate the *in vivo* potential of niclosamide analogs 11 and 32 and benzimidazole compounds SRI33576 and SRI35889, the compounds must be tested in animal studies. PK/PD studies investigating certain parameters such as bioavailability and metabolism should be conducted. It is not yet known whether the compounds are safe and efficacious in mouse tumor xenograft models.

Chemoresistance of certain cancers has been attributed to upregulated Wnt/β-catenin signaling. A study reported that docetaxel or doxorubicin treatment elicited nonsignificant impact on proliferation in two TNBC cell lines. In addition, both chemotherapies were found to lead to loss of regulation of genes linked to stemness in TNBC. Components of the Wnt/β-catenin signaling pathway, such as FZD co-receptors, Axin1, and Wnt5a, lead to activation of stem cell generation and renewal in differentiated

TNBC cells. In effect, Wnt/β-catenin signaling may confer acquired chemoresistance observed in refractory TNBC tumors [50].

Effective treatment of breast and ovarian cancers and prevention of subsequent relapse requires elimination of bulk tumor cells and CSCs. Since breast CSCs exhibit upregulated Wnt/β-catenin signaling activity compared to the bulk population, these cells should be further investigated for their sensitivity to compounds SRI33576 and SRI35889. The subpopulation of cancer cells known as CSCs exhibit higher Wnt/β-catenin signaling compared to non-CSCs. Such higher activity is known to mediate resistance to radiation [105], chemoresistance [106-108], and increased metastasis [109-112]. Thus, these cells are appealing targets for therapy of TNBC.

Both autocrine and paracrine signaling are implicated in proliferation of TNBCs. Green, et al. evaluated TNBC cell lines and found those of the basal B (claudin-low) type as significantly activated by Wnt/β-catenin signaling. The claudin-low phenotype is associated with EMT, and Wnt/β-catenin signaling is known to promote EMT [113]. Further investigation must be conducted to examine potential differential effects of the benzimidazole compounds on autocrine and paracrine Wnt/β-catenin signaling activities.

Clinical trials are currently investigating combinatorial Wnt antagonists and chemotherapy in breast and ovarian cancers. Vantictumab (OMP-18R5), a Wnt antagonist developed to obstruct binding of Wnt ligands to FZD- 1, -2, -5, -7, and -8, is undergoing investigation in combination with paclitaxel (NCT01973309) in metastatic breast cancer. Another antagonist, ipafricept (OMP-54F28), which prevents induction of Wnt/β-catenin signaling by sequestering secreted Wnt ligands, is undergoing investigation in combination with paclitaxel and carboplatin in ovarian cancer.

(NCT02092363). In animal studies, both agents inhibited tumor growth in patient-derived xenograft (PDX) models [114]. Combination therapy of the niclosamide 11 and 32 analogs or benzimidazole compounds SRI33576 and SRI35889 with chemotherapy may exhibit enhanced antitumor efficacy *in vivo*.

General Conclusions

Ovarian cancer and TNBC are characterized by upregulated Wnt/β-catenin signaling activity. The work accomplished in this dissertation is important as it identified novel compounds with cytotoxicity and Wnt/β-catenin-inhibiting efficacy against TNBC and ovarian cancer. WNT974 and new niclosamide analogs overcame chemoresistance in ovarian cancer to inhibit proliferation. Benzimidazole compounds were revealed as selective inhibitors of Wnt/β-catenin signaling in TNBC with comparably minimal effects against nonmalignant cells. These agents represent potentially effective therapies against both cancers *in vivo*, which would be the next logical step to investigate. They may also be effective against other Wnt/β-catenin-dependent cancers with potential to restrict relapse and metastasis. The structures of the agents presented in this dissertation may be used as templates for development of additional new compounds with improved pharmacokinetics and pharmacodynamics, and more selective Wnt/β-catenin signaling inhibition *in vivo*.

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APPENDIX A

IACUC APPROVAL FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: 08-Sep-2015

TO: Arend, Rebecca

FROM: Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 08-Sep-2015.

Protocol PI: Arend, Rebecca

Title: Treatment Of Ovarian Cancer With Wnt/ß-Catenin Inhibitors

Sponsor: UAB DEPARTMENT

Animal Project Number (APN): IACUC-20205

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

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