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A Novel Resveratrol-Salinomycin Combination Sensitizes ER-Positive Breast Cancer Cells to Apoptosis

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Running Title: Salinomycin enhances effects of resveratrol

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Conflict of Interest Statement

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Abstract

Background: Resveratrol is a dietary compound that has been widely reported for its anticancer activities. However, successful extrapolation of its effects to pre-clinical

studies is met with limited success due to inadequate bioavailability. We investigated the potential of combination therapy to improve the efficacy of resveratrol in a more physiologically relevant dose range.

Methods: The effect of resveratrol on canonical Wnt signaling was evaluated by Western blotting. Wnt modulators HLY78 (activator) and salinomycin (inhibitor) were evaluated in combination with resveratrol for their effect on breast cancer cell viability (MTT assay), cell cycle progression and apoptosis (Western blotting). Bliss independency model was used to evaluate combinatorial effects of resveratrol-salinomycin combination.

Results: Resveratrol downregulated canonical Wnt signaling proteins in treated breast cancer cells (MCF-7, MDA-MB-231 and MDA-MB-468) in the dose range of 50 to 200 µM, which also affected cellular viability. However, at very low doses (0-50 µM), resveratrol exhibited no cellular toxicity. Co-treatment with salinomycin significantly potentiated the anti-cancer effects of resveratrol, whereas HLY78 co-treatment had minimal effect. Bliss independency model revealed that Wnt inhibition synergistically potentiates the effects of resveratrol in MCF-7 cells. Significantly downregulated canonical Wnt signaling proteins and marker of epithelial-mesenchymal transition (EMT), vimentin were observed in cells treated with resveratrol-salinomycin combination. Cell cycle arrest, caspase activation and apoptosis induction in cells treated with resveratrol-salinomycin combination further confirmed the efficacy of the combination.

Conclusion: We report a novel resveratrol-salinomycin combination for targeting ER-positive breast cancer cells and present evidence for successful pre-clinical

implementation of resveratrol.

Key words: Breast cancer, Resveratrol, HLY78, Salinomycin, Apoptosis

Introduction

Breast cancer is the leading cause of cancer-related deaths in women in the United States (US), with an estimated 246,660 new cases of breast cancer diagnosed in the year 2016 in US (http://www.breastcancer.org/symptoms/understand_bc/statistics). The existence of different types of breast cancers such as estrogen receptor (ER)-positive breast cancer and triple-negative breast cancers among women presents complex treatment challenges as drugs that target ER-positive breast cancer fails to exhibit similar efficacies in triple-negative breast cancers and *vice versa* [1-2]. Significant interest has been generated in development of drugs from medicinal plants and several plant-derived agents including resveratrol are currently in clinical trials for their efficacy against colon cancer, gastrointestinal cancer and follicular lymphoma (ClinicalTrial.gov).

Resveratrol, a plant polyphenol is reported to hold promising chemopreventive and pro-apoptotic properties against several cancers [3-6]. Despite its promising therapeutic effects, there exist inherent issues with bioavailability of resveratrol that may be attributed to its structure, chemical properties and several other factors such as the site of metabolism, tissue accumulation and the activity of deconjugation enzymes [7]. Available published reports of the efficacy of resveratrol in inducing cell death in dose ranges of 50-200 μM in *in vitro* studies, are not reproducible *in vivo* due to bioavailability issues in animals [3-4, 6, 8]. Alternative approaches to enhance bioavailability and efficacy of resveratrol *in vivo* have been proposed by several investigators. For instance,

Johnson *et al*. reported enhanced bioavailability of resveratrol in combination with piperine [9]. Subsequently, piperine was also found to potentiate the effects of resveratrol in cellular and animal models [10-11]. Similarly, Goldberg *et al*. observed improved bioavailability when resveratrol was co-administered with grape juice, V8 juice or red wine [12]. While a resveratrol dose ranges of 1-10 μM is closer to being successfully achievable *in vivo*, there are very few reports on the anticancer effects of low dose resveratrol *in vitro* [13-15].

Signal transduction pathways are investigated extensively for their roles in the etiology of breast cancer and various signaling pathways including MAPK, PI3K and canonical Wnt signaling pathways are reported to play a key role in breast cancer [16-19]. The canonical Wnt signaling is activated by binding of Wnt ligand to receptor Frizzled (Fz) and its co-receptor Low Density Lipoprotein Receptor-Related Protein 6 (LRP6). This binding activates proteins belonging to the family Dishevelled (Dvl) leading to β-catenin accumulation and activation of T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors [20-21]. The first successful phase I clinical trial study involving resveratrol identified Wnt signaling as a key target and highlighted this signaling cascade as a viable target for resveratrol action [22]. Resveratrol is reported to downregulate canonical Wnt signaling in colon cancer cells without exhibiting cellular toxicity or inducing apoptosis [23]. Involvement of the canonical Wnt signaling and epithelial mesenchymal transition (EMT) in breast cancer is also reported [24]. Interestingly, there are no reports focusing on complementing resveratrol activity by modulating Wnt signaling.

In this study, we investigated the effect of resveratrol and Wnt modulators on ER-

positive (MCF-7 and BT474) and triple negative (MDA-MB-231 and MDA-MB-468) breast cancer cells. Combination treatment including resveratrol and Wnt inhibitor, salinomycin, synergistically potentiated cellular toxicity and apoptosis inducing effects of resveratrol, specifically in ER-positive breast cancer cells. Overall, the study identifies a novel resveratrol-salinomycin combination that could overcome the shortcomings of utilizing resveratrol alone and could pave the way for successful pre-clinical implementation of this promising anticancer drug.

Materials and Methods

Chemicals and reagents. Resveratrol was obtained from Sigma-Aldrich (St. Louis, MO, USA). A 100 mM stock was prepared in ethanol and stored at 4°C until further use. All antibodies including caspase-8, caspase-9, cyclin-dependent kinases (CDKs) - 2 and 4, Low-density lipoprotein receptor-related protein 6 (LRP6), Wnt5AB, Dishevelled Segment Polarity Protein 2 (Dvl2), β-catenin, Vimentin, HRP-conjugated anti-rabbit IgG and anti-mouse IgG antibodies was purchased from Cell Signaling Technology (Danvers, MA, USA). B-cell lymphoma 2 (Bcl2) antibody was from Santa Cruz Biotechnology (Dallas, TX, USA). β-actin antibody and 4-Ethyl-5,6-Dihydro-5-methyl-[1,3]dioxolo[4,5-j]phenanthridine,4-Ethyl-5-methyl-5,6-dihydro-[1,3]dioxolo[4,5-j]phenanthridine (HLY78) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Salinomycin Sodium Salt was from MP Biomedicals (Santa Ana, CA, USA). Rapamycin was from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). The remaining chemicals and solvents used were of standard analytical grade and HPLC grade respectively.

Cell culture. Human breast adenocarcinoma cells MCF-7, MDA-MB-231, MDA-MB-

468 and human breast ductal carcinoma cell BT474 were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle medium (Thermo Scientific, Waltham,MA, USA) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin in a 5% CO_2 environment at 37°C. All treatments were performed in serum free medium unless mentioned otherwise.

MTT assay. Breast cancer cells were treated with indicated concentrations of resveratrol, HLY78, salinomycin, rapamycin or drug combinations for 24 hours. MTT assay was performed as described before [25].

Bliss Independency Model. MTT assays were performed in breast cancer cells with varying drug combinations and cellular toxicity was determined relative to untreated control cells. The Bliss model was used to quantify the effect of resveratrol combinations with Wnt activator (HLY78) and Wnt inhibitor (salinomycin). This model computes the expected combined effects of two drugs as the product of their individual effects. The drug combinations are: synergistic if the observed effects of drug combinations are greater than the expected combined effects; antagonistic if the observed effects of drug combinations are lesser than the expected combined effects and additive if the observed effects of drug combinations are equal to the expected effects [26]. For the current study, a bliss value $\geq$1.1 was considered synergy and $\leq$ 0.95 was considered antagonism.

Western blot analysis. Cell lysates were resolved on a 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting performed as described before [25].

Caspase 8/9 activity assay. Caspase -8 and -9 activities were detected using

CaspGLOW™ Fluorescein Active Caspase -8 and -9 staining kits (BioVision, Milpitas, CA, USA) according to the manufacturer's instructions.

Scratch assay for cell migration. Cells were seeded in 12-well plates and a 1 ml pipette tip was used to scratch sub-confluent cultures for the scratch assay. Cells were washed with PBS and treated for 48 hours in complete medium. Bright field pictures were taken at 0 and 48 hours and relative cell migration was quantified using ImageJ software (Java image processing, NIH).

Statistical analysis. Representative data from three or more independent experiments are shown as mean value ± SEM. Statistical analysis was performed with two-way analysis of variance to identify differences between groups using GraphPad Prism Software (San Diego, CA, USA) and p values <0.05 considered significant.

Results

Resveratrol downregulates canonical Wnt signaling in breast cancer cells.

MCF-7, MDA-MB-231 and MDA-MB-468 breast cancer cells were treated with resveratrol at a dose range (50-200 μM) usually tested *in vitro* and protein components of the canonical Wnt signaling pathway were investigated. Significant downregulation of canonical Wnt signaling was observed with resveratrol treatment on all breast cancer cells. The lowest dose of resveratrol (50 μM) significantly decreased expression levels of LRP6 and β-catenin, which are the key upstream (LRP6) and downstream (β-catenin) proteins of the canonical Wnt signaling (Figure 1). We evaluated the cellular toxicity of resveratrol (0-200 μM) on four breast cancer cell-lines. Resveratrol did not exhibit any significant toxic effects at doses below 50 μM (Supplementary figure 1).

Effect of HLY78 and salinomycin on canonical Wnt signaling in breast cancer cells.

We used specific modulators of canonical Wnt signaling to investigate the combinatorial effects of low dose resveratrol in breast cancer cells. HLY78 is a commercially available specific activator and salinomycin is a commercially available specific inhibitor of the Wnt/β-Catenin signaling pathway. HLY78 upregulated canonical Wnt signaling proteins LRP6, Wnt5AB, Dvl2 and β-catenin proteins in treated MCF-7, MDA-MB-231 and MDA-MB-468 cells in a dose-dependent manner (Figure 2A). The only discrepancy was in the effect on Wnt 5AB protein in MDA-MB-231 cells, where higher doses downegulated the protein. The Wnt inhibitor, salinomycin, downregulated canonical Wnt signaling proteins in a dose-dependent manner in all three-breast cancer cell lines (Figure 2B). The effects observed for Wnt5AB, however, was less pronounced in MDA-MB-231 and MDA-MB-468 with only the highest dose significantly downregulating the protein. Similarly, the highest dose caused a slight upregulation in Dvl2 and β-catenin proteins in MDA-MB-468 cells. These discrepancies could be due to cell line specificity, off target effects and the involvement of other Wnt related proteins.

Combinatorial effect of resveratrol and Wnt activator HLY78

MTT assay was performed and bliss-independency model was used to investigate the net effect of the combination of resveratrol and Wnt activator HLY78 on cell viability. Resveratrol at a low dose (10 μM) did not significantly affect cell viability whereas HLY78 displayed marginal cellular toxicity in treated cells. Interestingly, the combination of HLY78 and resveratrol showed minimal effects as compared to the control in all four cell-lines (Figures 3A-D). The bliss-independency model predicted an antagonistic effect for the combination of resveratrol and Wnt activator in all four-breast

cancer cells (Figure 3E).

Combinatorial effects of resveratrol and Wnt inhibitor salinomycin

MTT assay was performed and bliss-independency model was used to investigate the net effect of the combination of resveratrol-salinomycin on all breast cancer cell lines. Salinomycin treatment showed minimal effect on cell viability in MCF-7 and MDA-MB-231 cells and exhibited significant cellular toxicity to BT474 and 468 cells as compared to untreated and resveratrol control. Co-treatment of salinomycin with resveratrol had significant potentiated effect in MCF-7 and BT474 cells (Figure 4A-B). In both triple-negative breast cancer cell-lines, salinomycin treatment alone or in combination with resveratrol showed similar effects on cell viability (Figures 4C-D). The bliss-independency model predicted that the combination of resveratrol (10 μM) and salinomycin (200 nM) exhibited a synergistic effect in MCF-7 and BT474 cells, an additive effect in MDA-MB-231 cells and an antagonistic effect in MDA-MB-468 cells (Figure 4E).

Resveratrol-salinomycin combination enhances downregulation of canonical Wnt signaling and EMT marker in ER-positive breast cancer cells.

MCF-7 and BT474 cells were treated with resveratrol individually and in combination with salinomycin and expression of proteins of the canonical Wnt signaling pathway were analyzed by Western blotting. Resveratrol-salinomycin combination significantly downregulated protein components of the canonical Wnt signaling as compared to resveratrol treatment (Figure 5A-B). Also, MCF-7 cells treated with the combination exhibited decreased levels of the EMT marker, vimentin. Both cell lines showed significant upregulation of E-Cadherin, further confirming the potential of the identified

combination in inhibiting EMT.

Resveratrol-salinomycin combination sensitizes ER-positive breast cancer cells to cell cycle arrest and apoptosis.

MCF-7 and BT474 cells were treated with the resveratrol-salinomycin combination and evaluated for cell cycle arrest, caspase activation and apoptosis induction by Western blotting. Resveratrol-salinomycin co-treatment downregulated CDK2 and CDK4 proteins as compared to resveratrol treated cells indicating a possible G-phase cell cycle arrest (Figure 6A-B). Resveratrol-salinomycin co-treatment induced significant downregulation of pro-apoptotic proteins including PARP, caspase -8, and -9 in MCF-7 and BT474 cells as compared to resveratrol treatment (Figure 6). Furthermore, levels of anti-apoptotic protein Bcl2 was decreased by resveratrol-salinomycin co-treatment in MCF-7 cells (Figure 6A). Caspase -8 and -9 activity assay further confirmed the apoptosis induction abilities of the resveratrol-salinomycin combination in MCF-7 and BT474 cells (Figure 7A-B). The combination inhibited cell migration in both cell lines (Figure 7C-D and supplementary figure 2). We also tested a combination of resveratrol with rapamycin and observed no significant enhancement in the effects of low dose resveratrol (Figure 7A-B).

Discussion

Improving the bioavailability and efficacy of resveratrol for better therapeutic outcomes has gained significant interest in recent years. While existing reports on clinical trials indicates that the bioavailability of resveratrol in *in vivo* systems continues to remain poor [27-28], promising increase of resveratrol in plasma and tissue are observed

in animal studies [29-31]. The Wnt signaling pathway was observed to be downregulated in the first successful phase I clinical trial that aimed at investigating the cancer preventive effects of resveratrol [22]. We validated the effect of resveratrol treatment on canonical Wnt signaling in ER-positive (MCF-7 and BT474) and triple negative (MDA-MB-231 and MDA-MB-468) breast cancer cells. Resveratrol treatment in a range usually tested *in vitro* (50-200 μM), caused significant downregulation of key protein components involved in the canonical Wnt signaling pathway (Figure 1). Downregulation of canonical Wnt signaling was observed at a dose of 50 μM which exhibited no significant cellular toxicity or apoptosis (Supplementary figure 1) [25]. This was in line with the study of Hope *et al*. who reported similar observations in colon cancer [23].

ER-positive breast cancer is observed to have a better five-year survival rate after diagnosis compared to triple-negative breast cancers [32]. However, the survival rate after five years continues to remain poor for both ER-positive and triple-negative breast cancer [33-34]. Combination experiments involving resveratrol and other plant components have yielded interesting anticancer effects in both *in vitro* and *in vivo* systems [9, 35-36]. There are reports on the effects of Wnt modulators in combination with anticancer agents to target various cancers [37-39]. We tested the effect of Wnt modulators, HLY78 (Wnt activator) and salinomycin (Wnt inhibitor) on canonical Wnt signaling in breast cancer cells (Figure 2). HLY78 in combination with low dose of resveratrol did not enhance the observed cellular toxicity in breast cancer cells and the bliss model predicted an antagonistic net effect of resveratrol-HLY78 combination in all four breast cancer cell-lines indicating the importance of canonical Wnt signaling in resveratrol mediated effect (Figure 3). Resveratrol-salinomycin combination, on the other

hand, significantly enhanced cellular toxicity of low dose resveratrol in MCF-7 and BT474 cells (Figure 4A-B) and the Bliss model predicted a synergistic effect for the combination (Figure 4E). Similar potentiation of cellular toxicity of resveratrol was not achieved in treated MDA-MB-231 and MDA-MB-468 cells. The bliss model predicted an antagonistic effect for the combination in MDA-MB-468 cells, and an additive effect in MDA-MB-231 cells. While, it can be concluded that the canonical Wnt signaling also plays a key role in MDA-MB-231 cells, the predicted additive effects, unlike synergistic effect observed in MCF-7 cells, requires careful evaluation of the involvement of other signaling pathways, if any, to delineate the underlying cellular regulation in these cells. Overall, the data indicated that resveratrol-salinomycin combination was particularly effective in ER-positive breast cancer cells.

In addition to downregulating protein components of the canonical Wnt signaling in MCF-7 and BT474 cells, treatment with the resveratrol-salinomycin combination resulted in significant downregulation of the EMT marker vimentin and upregulation of E-Cadherin, a well-known inhibitor of EMT (Figure 5). These results suggest that the resveratrol-salinomycin combination could modulate Wnt/EMT signaling in ER-positive breast cancer cells. Cell cycle arrest and induction of apoptosis are key aspects of cancer therapy and our group has recently reported that the anticancer effects of resveratrol are mediated by downregulation of key proteins involving CDKs specific for G-phase arrest, anti-apoptotic proteins belonging to the inhibitor of apoptosis (IAPs) family of proteins Bcl2 and XIAP, and induction of apoptosis in treated MCF-7 and MDA-MB-231 breast cancer cells [25]. We now report enhanced anticancer potential of low dose resveratrol in ER-positive breast cancer cells. We observe that treatment of MCF-7 and BT474 cells

with resveratrol-salinomycin combination downregulates CDK2 and CDK4 proteins indicating G-phase cell cycle arrest and induces apoptosis (Figure 6). The combination significantly downregulated anti-apoptotic protein Bcl2 in MCF-7 cells (Figure 6). Significant caspase -8 and -9 activity and inhibition of cell migration were confirmed in cells treated with the combination (Figure 7). The combination of resveratrol with salinomycin was observed to be more potent that a combination with rapamycin, a widely used mTOR inhibitor, indicating that the resveratrol-salinomycin combination could yield better therapeutic effects than currently used strategies to target ER-positive breast cancer (Figure 7).

In summary, our study identifies a novel resveratrol-salinomycin combination that targets breast cancer cells. The resveratrol-salinomycin combination targets breast cancer cells by downregulating canonical Wnt/EMT signaling; inducing cell cycle arrest and apoptosis. The study presents key aspects of the mechanism of action of resveratrol in combination with Wnt signal transduction modulators in breast cancer. The study highlights the importance of testing combination of drugs such as resveratrol with signal transduction modulators that could potential enhance the cytotoxic effects and will be the key for successful clinical translation of this promising anticancer agent.

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Figure legends:

Figure 1: Regulation of canonical Wnt signaling by resveratrol. MCF-7, MDA-MB-231 and MDA-MB-468 cells were treated with the indicated concentrations of resveratrol for 24 hours, and then probed for canonical Wnt proteins LRP6, Wnt5AB, Dvl2 and β-catenin. Blots were reprobed with β-actin antibody to confirm equal loading of the

samples. Representative blots from three independent experiments are shown. The immunoblot signals were quantified by densitometry. Plots are mean ± SEM (n=3). *p<0.05 *versus* non-treated control.

Figure 2: Regulation of canonical Wnt signaling by HLY78 and salinomycin in breast cancer cells. MCF-7, MDA-MB-231 and MDA-MB-468 cells were treated with the indicated concentrations of A) HLY78 and B) salinomycin for 24 hours, and then probed for canonical Wnt proteins LRP6, Wnt5AB, Dvl2 and β-catenin. Blots were reprobed with β-actin antibody to confirm equal loading of the samples. Representative blots from three independent experiments are shown. The immunoblot signals were quantified by densitometry. Values are mean ± SEM (n=3). *p<0.05 *versus* non-treated control.

Figure 3: Combinatorial effect of resveratrol and Wnt activator HLY78. A) MCF-7, B) BT474, C) MDA-MB-231 and D) MDA-MB-468 cells were treated with resveratrol (10 μM), HLY78 (50 nM) and combinations of resveratrol-HLY78 for 24 hours and cell viability was assessed by MTT assay. Plots are mean ± SEM (n=3). *p<0.05 *versus* untreated control. E) Combinatorial effects of resveratrol-HLY78 predicted by Bliss independency model for the four breast cancer cell-lines. Data represent mean values ± SEM of triplicate determinations from three independent experiments.

Figure 4: Combinatorial effects of resveratrol and Wnt inhibitor salinomycin. A) MCF-7, B) BT474, C) MDA-MB-231 and D) MDA-MB-468 cells were treated with resveratrol (10 μM), salinomycin (Sal) (200 nM) and combinations of resveratrol-salinomycin for 24 hours and cell viability was assessed by MTT assay. Plots are mean ± SEM (n=3). *p<0.05 *versus* non-treated control. #p<0.05 *versus* resveratrol-treated cells.

E) Combinatorial effects of resveratrol-salinomycin predicted by Bliss independency model for the four breast cancer cell-lines. Data represent mean values ± SEM of triplicate determinations from three independent experiments.

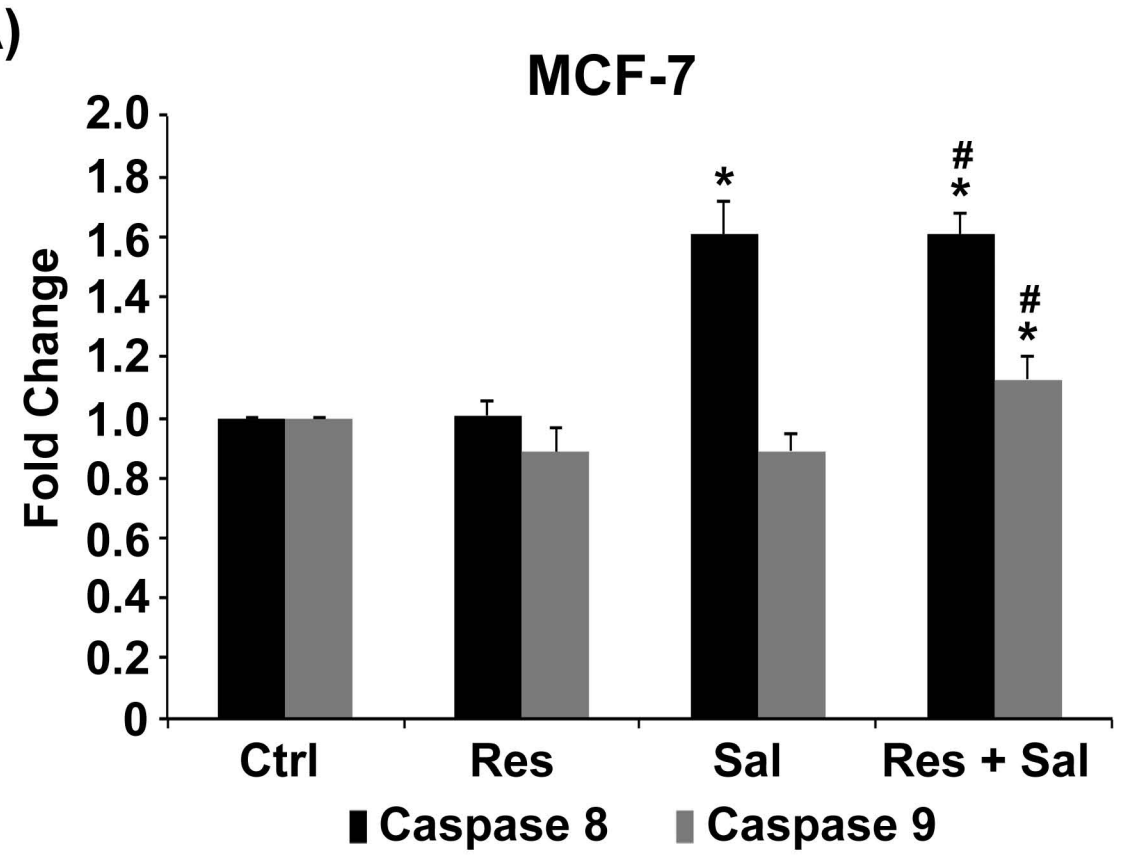
Figure 5: Resveratrol-salinomycin combination regulates canonical Wnt signaling in ER-positive breast cancer cells. Cell lysates from A) MCF-7 and B) BT474 cells left untreated or treated with resveratrol (10 μM), salinomycin (200 nM) or a combination of both for 24 hours were analyzed for canonical Wnt proteins (LRP6, Wnt5AB, Dvl2 and β-catenin) and EMT markers Vimentin and E-Cadherin. Blots were reprobed with β-actin antibody to confirm equal loading of the samples. Representative blots from three independent experiments are shown. The immunoblot signals were quantified by densitometry. Plots are mean ± SEM (n=3). **p*<0.05 *versus* non-treated control. #*p*<0.05 *versus* resveratrol-treated cells.

Figure 6: Resveratrol-salinomycin combination induces cell cycle arrest and apoptosis in breast cancer cells. Cell lysates from A) MCF-7 and B) BT474 cells left untreated or treated with resveratrol (10 μM), salinomycin (200 nM) or a combination of both for 24 hours were analyzed for G-phase cell cycle checkpoint proteins (CDK2 and CDK4), apoptotic proteins (PARP, cleaved PARP, Caspase-8 and -9) and anti-apoptotic protein Bcl2 by Western blotting. Representative data from three independent experiments are shown. Blots were reprobed with β-actin antibody to confirm equal loading of the samples. The immunoblot signals were quantified by densitometry. Plots are mean ± SEM (n=3). **p*<0.05 *versus* non-treated control. #*p*<0.05 *versus* resveratrol-treated cells.

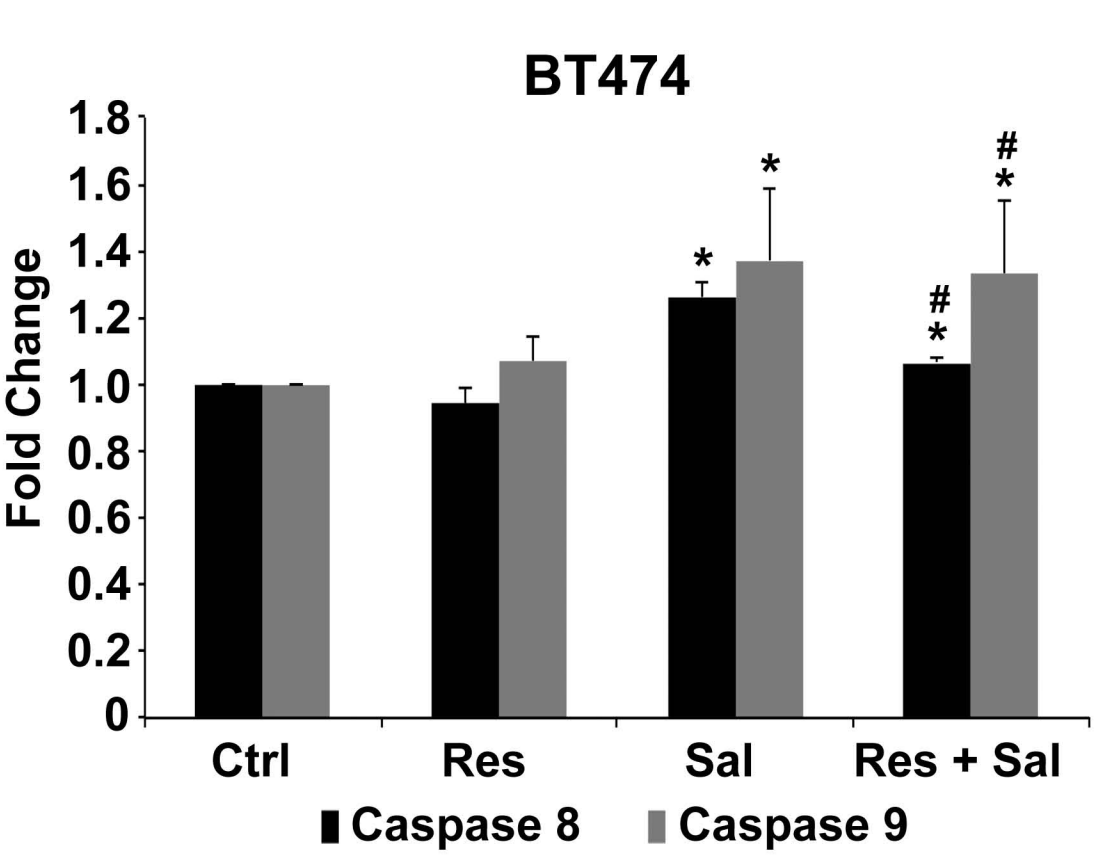
Figure 7: Resveratrol-salinomycin combination induces apoptosis and inhibits cell migration in breast cancer cells. A) MCF-7 and B) BT474 cells left untreated or treated

with resveratrol (10 μM), salinomycin (200 nM) or a combination of both for 24 hours were trypsinized and analyzed for Caspase -8 and -9 activities using CaspGLOW™ Fluorescein Active Caspase -8 and -9 staining kits. Plots are mean values ± SEM of duplicate determinations from two independent experiments. *p<0.05 *versus* non-treated control. #p<0.05 *versus* resveratrol-treated cells. Subconfluent C) MCF-7 and D) BT474 cells left untreated or treated with resveratrol (10 μM), salinomycin (200 nM) or a combination of both for 48 hours in complete medium were analyzed for relative cell migration by *in vitro* scratch assay. Plots are mean ± SEM (n=4). *p<0.05 *versus* non-treated control. #p<0.05 *versus* resveratrol-treated cells. E) MCF-7 and F) BT474 cells were treated with resveratrol (10 μM), rapamycin (Rap) (200 nM) and combinations of resveratrol-rapamycin for 24 hours and cell viability was assessed by MTT assay. Plots are mean ± SEM (n=3). *p<0.05 *versus* untreated control.

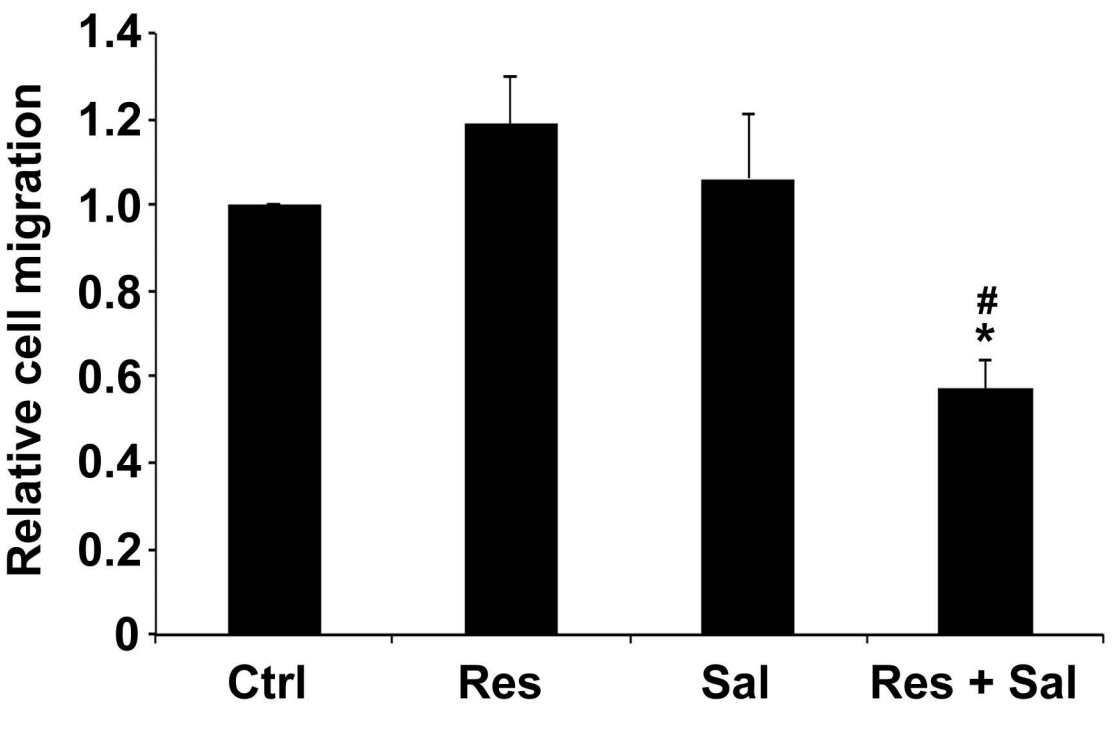
A)


MCF-7
Fold Change
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
#
*
#
*
Ctrl
Res
Sal
Res + Sal
Caspase 8
Caspase 9

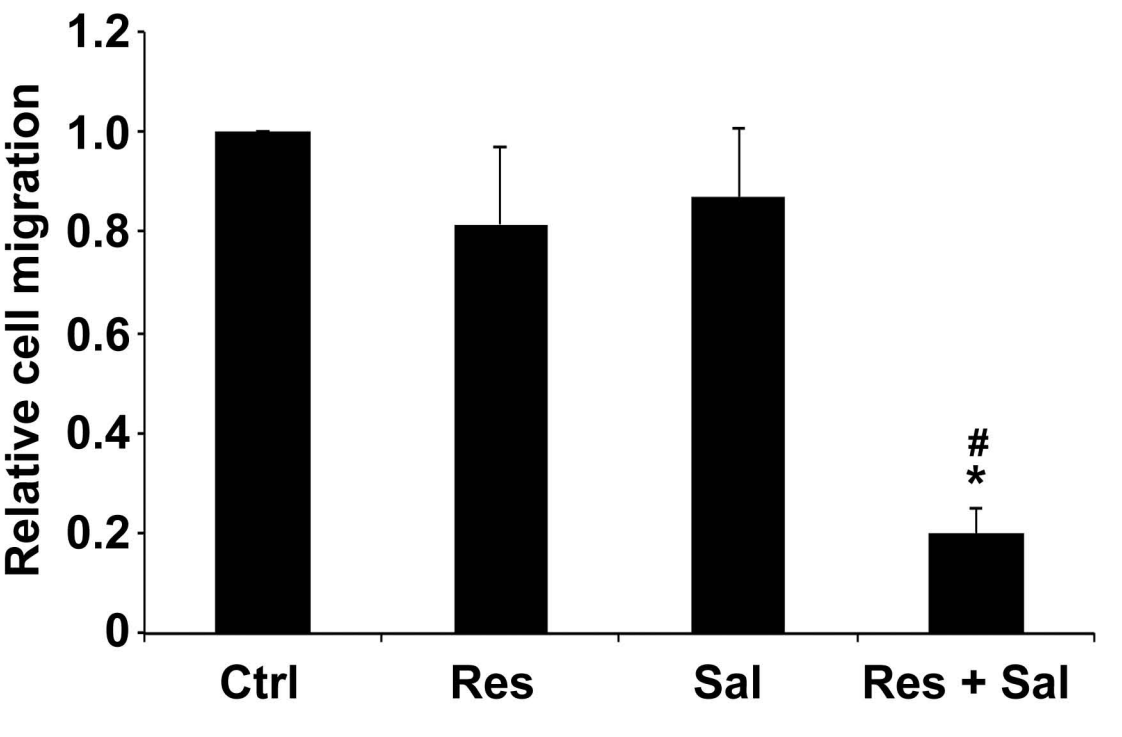
B)


BT474
Fold Change
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
*
#
*
Ctrl
Res
Sal
Res + Sal
Caspase 8
Caspase 9

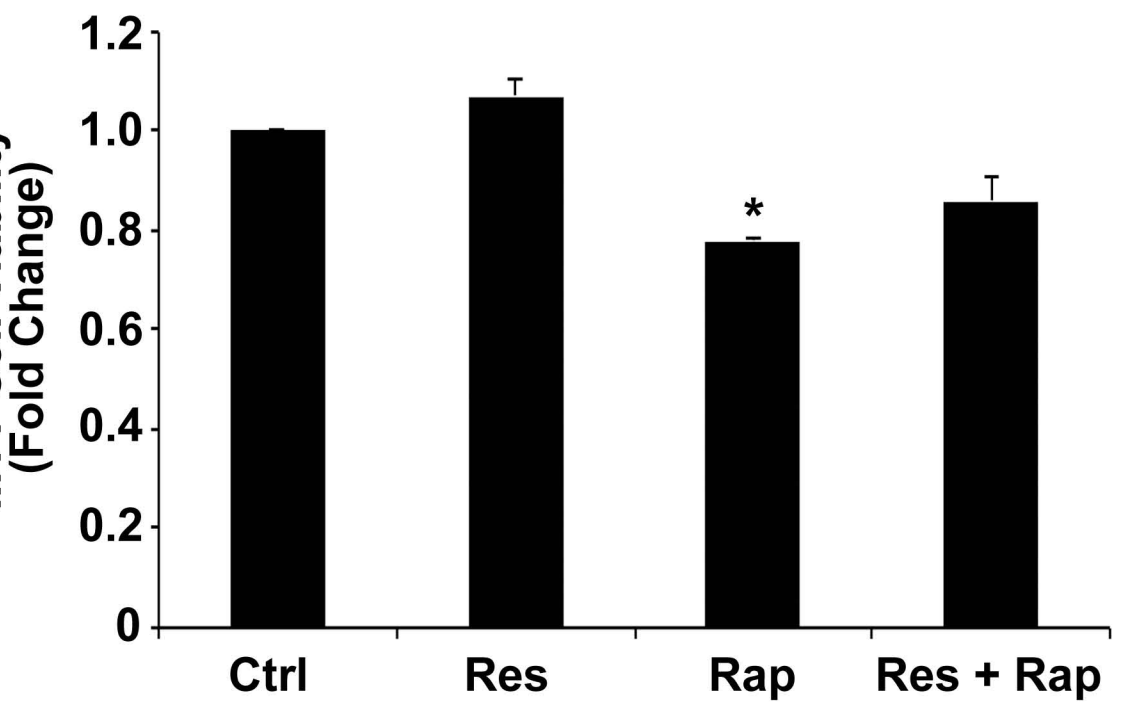
C)


Relative cell migration
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
#
*
Ctrl
Res
Sal
Res + Sal

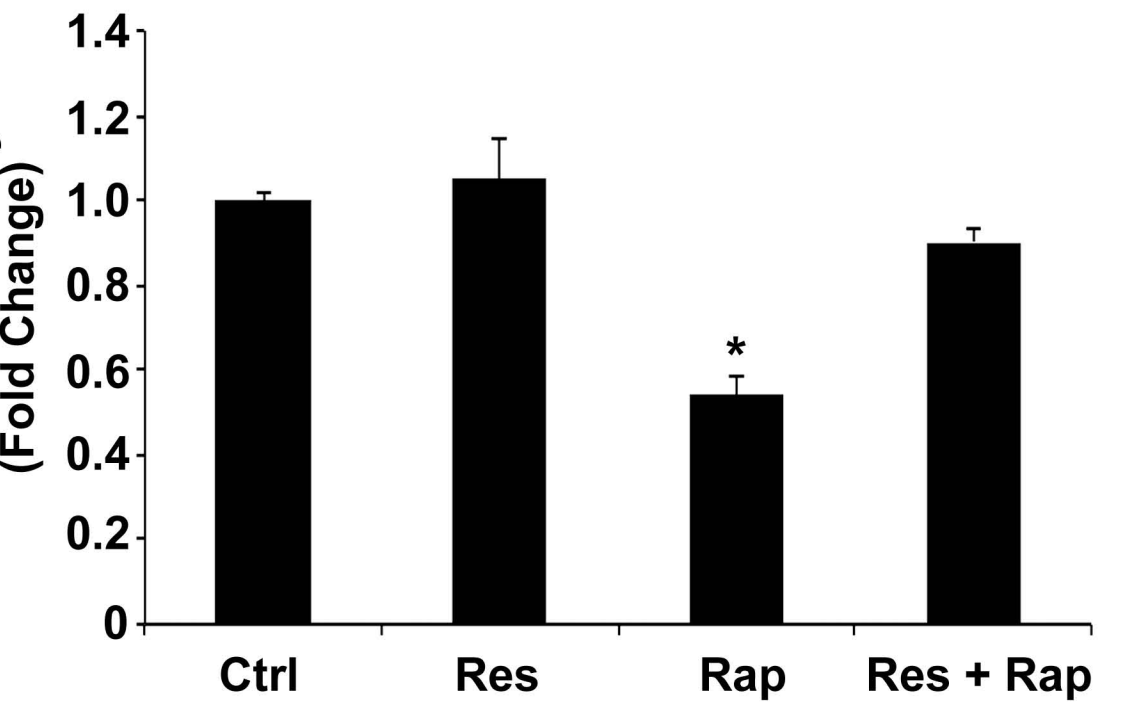
D)


Relative cell migration
1.2
1.0
0.8
0.6
0.4
0.2
0
#
*
Ctrl
Res
Sal
Res + Sal

E)


MTT Cell Viability
(Fold Change)
1.2
1.0
0.8
0.6
0.4
0.2
0
*
Ctrl
Res
Rap
Res + Rap

F)


MTT Cell Viability
(Fold Change)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
Ctrl
Res
Rap
Res + Rap

A)

MCF-7

- + - + Res (10 μM)

- - + + Sal (200 nM)

CDK2

CDK4

PARP

Cleaved PARP

Caspase 8

Caspase 9

Bcl2

β-actin

B)

BT474

- + - + Res (10 μM)

- - + + Sal (200 nM)

CDK2

CDK4

PARP

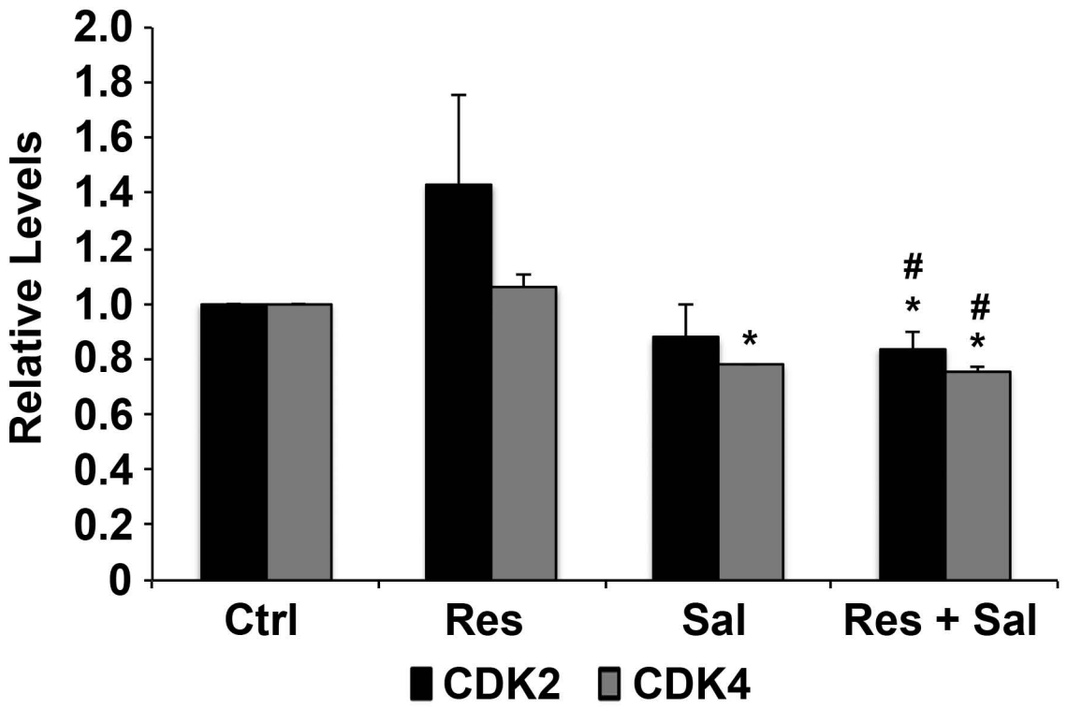
Cleaved PARP

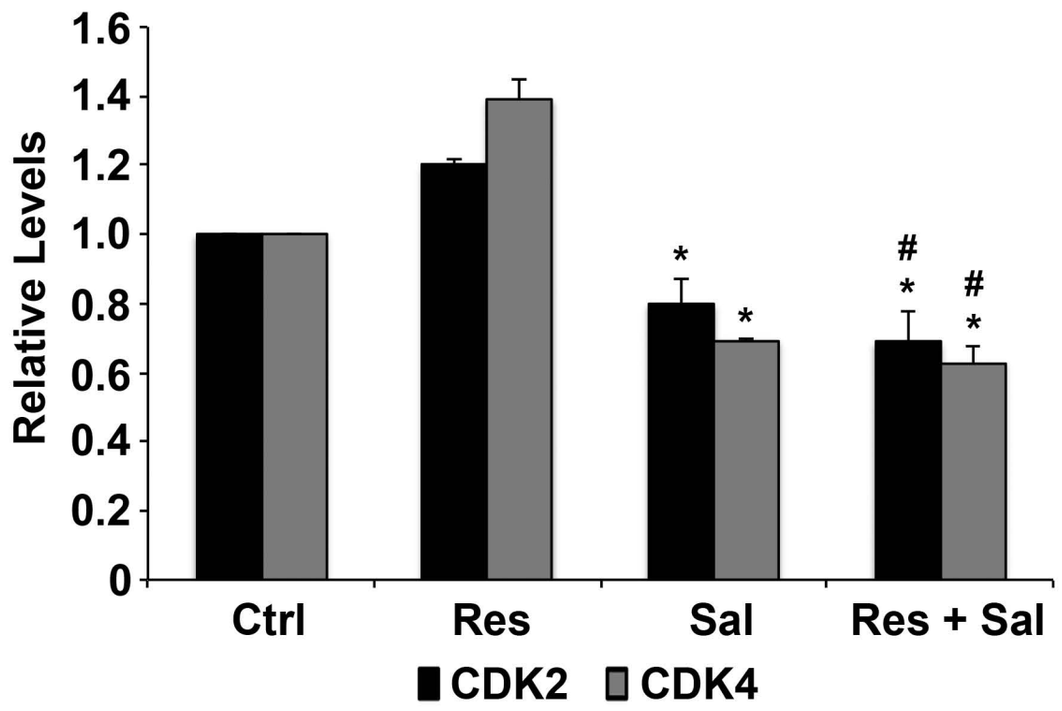
Caspase 8

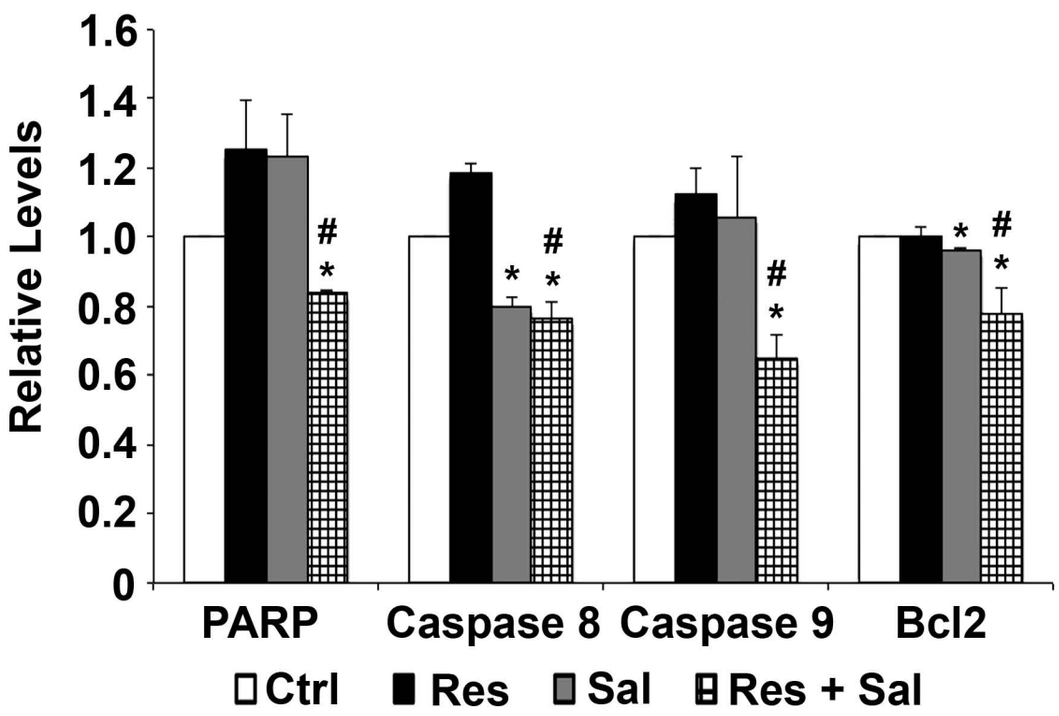
Caspase 9

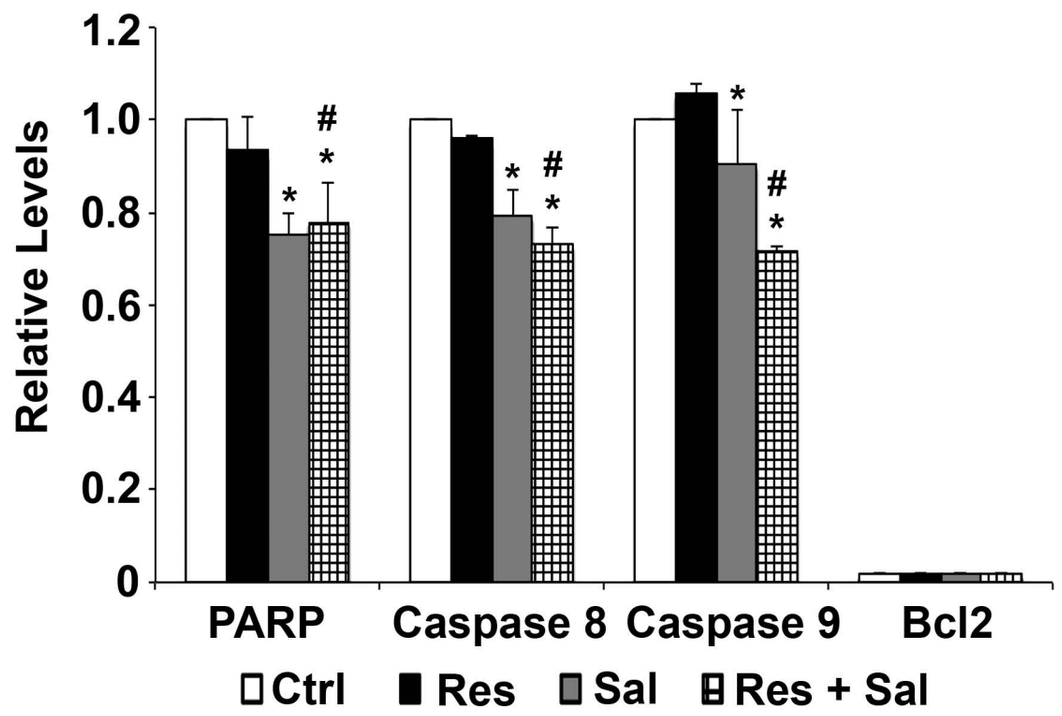
Bcl2

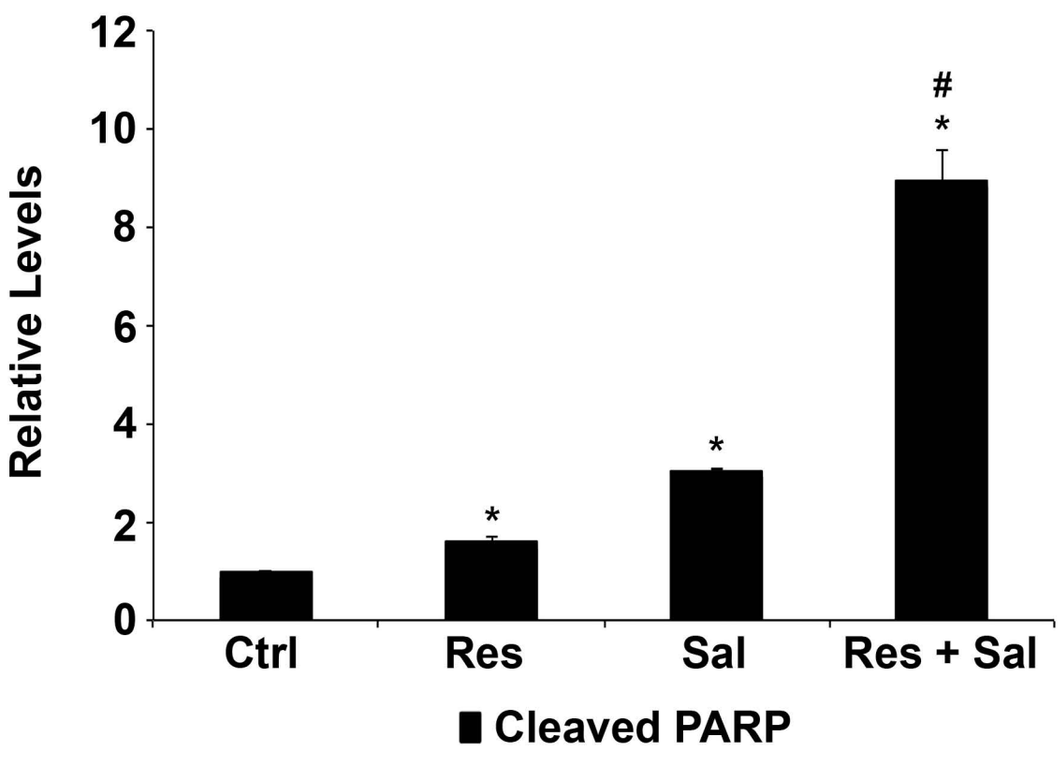
β-actin

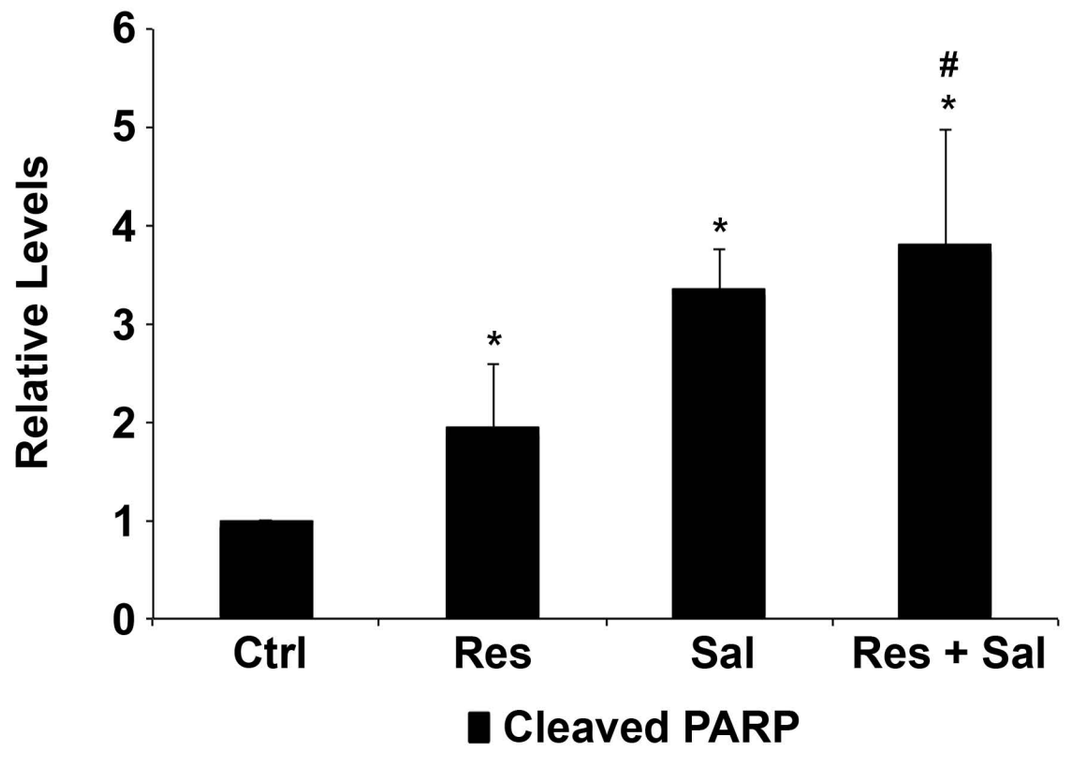












A)

MCF-7

- + - + Res (10 μM)
- - + + Sal (200 nM)

LRP6

Wnt5AB

Dvl2

β-catenin

Vimentin

E-Cadherin

β-actin

B)

BT474

- + - + Res (10 μM)
- - + + Sal (200 nM)

LRP6

Wnt5AB

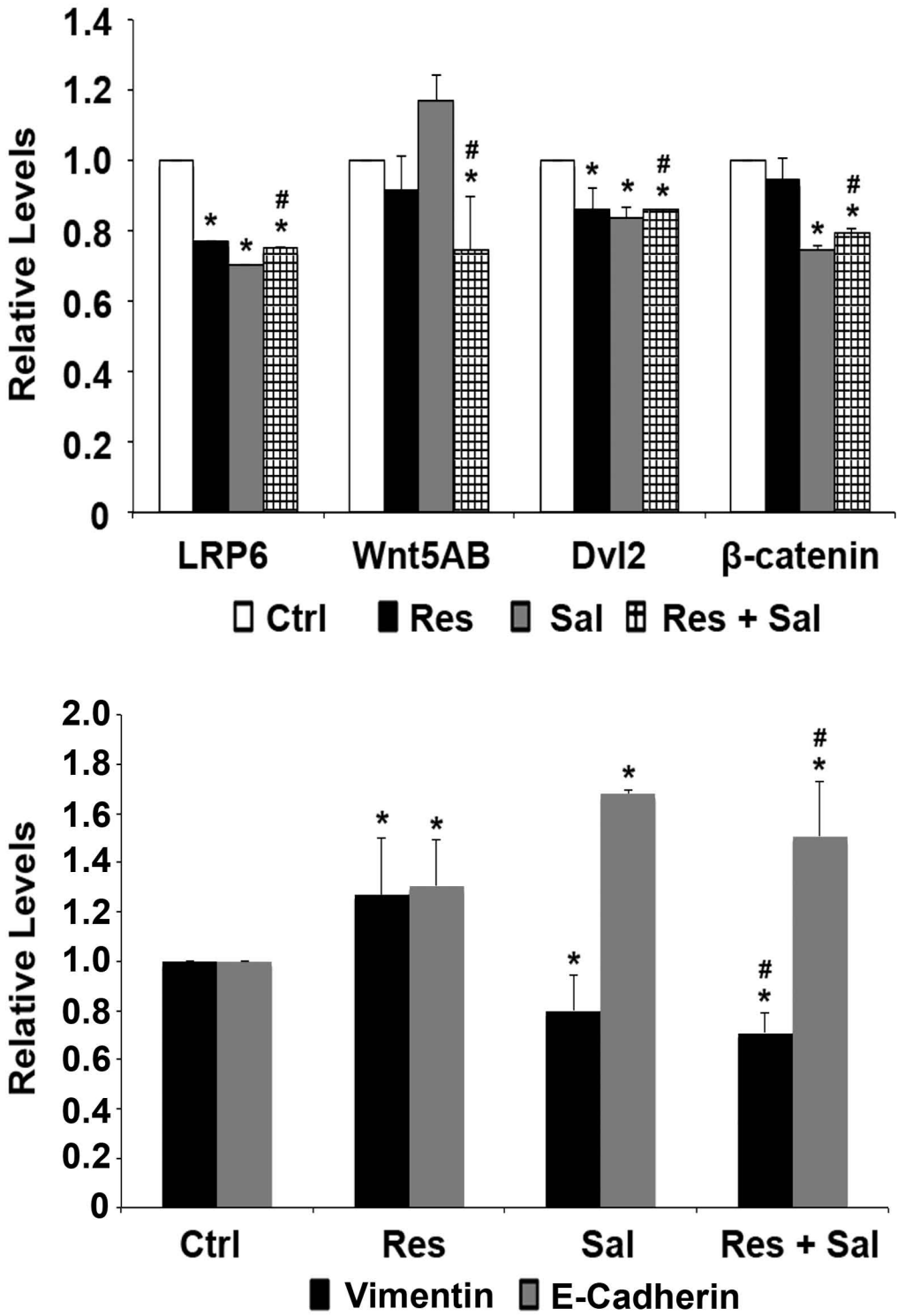
Dvl2

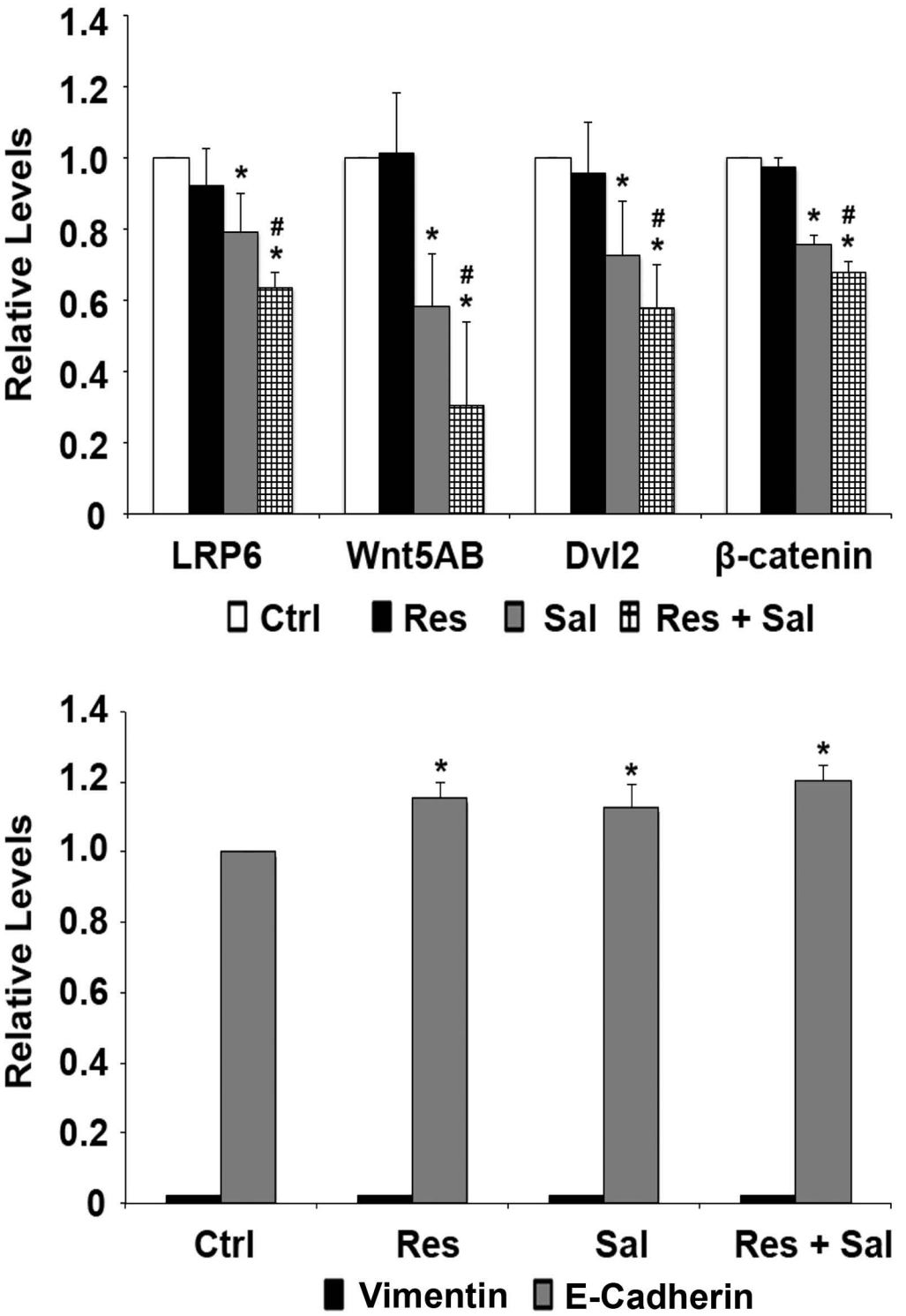
β-catenin

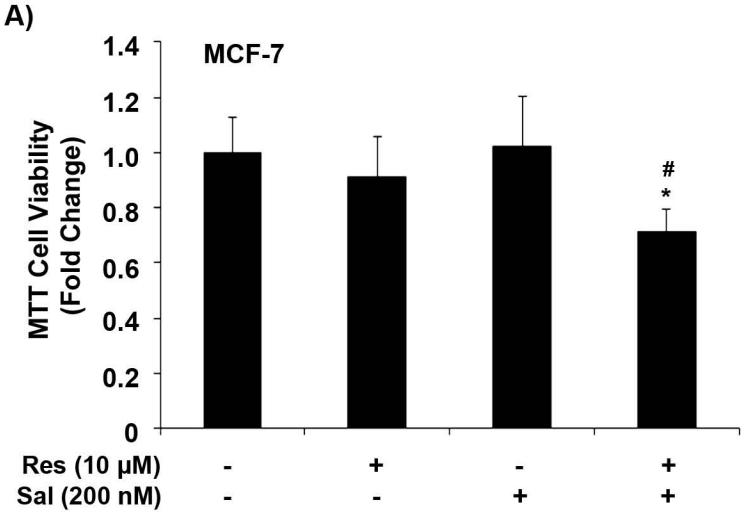
Vimentin

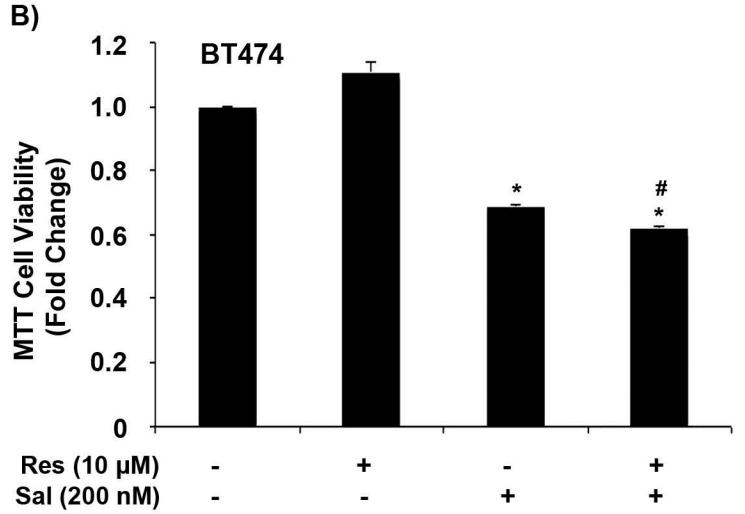
E-Cadherin

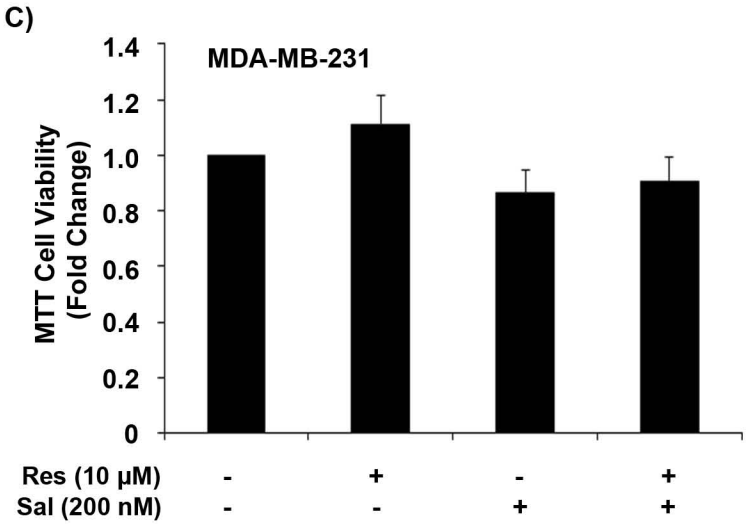
β-actin

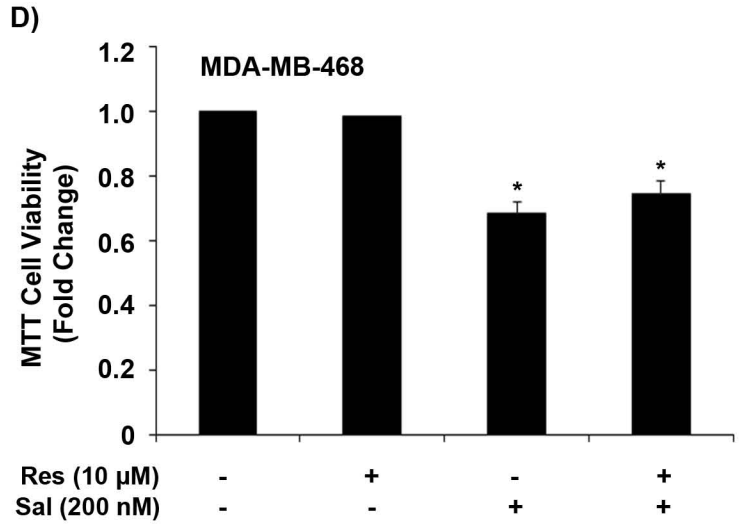






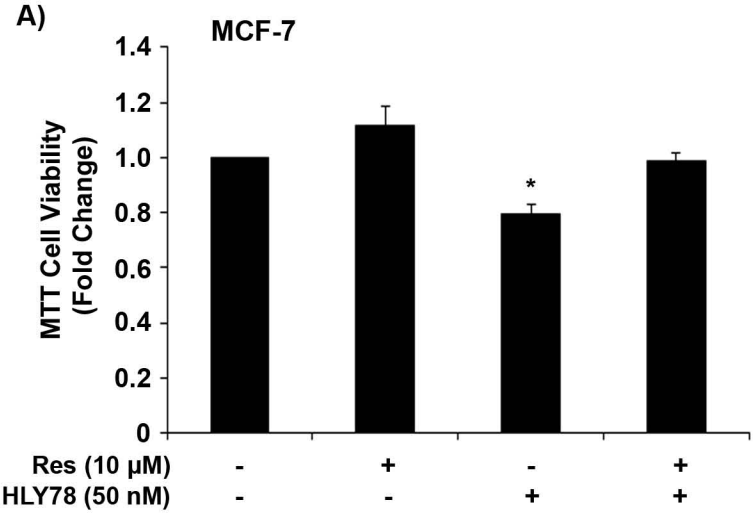


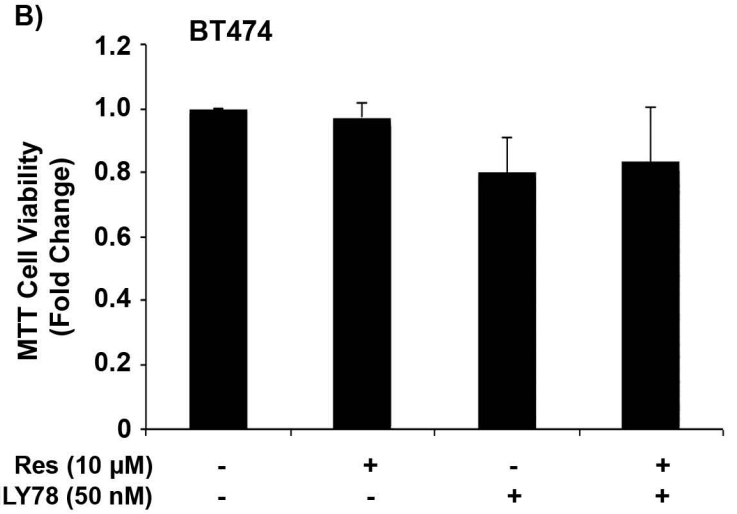


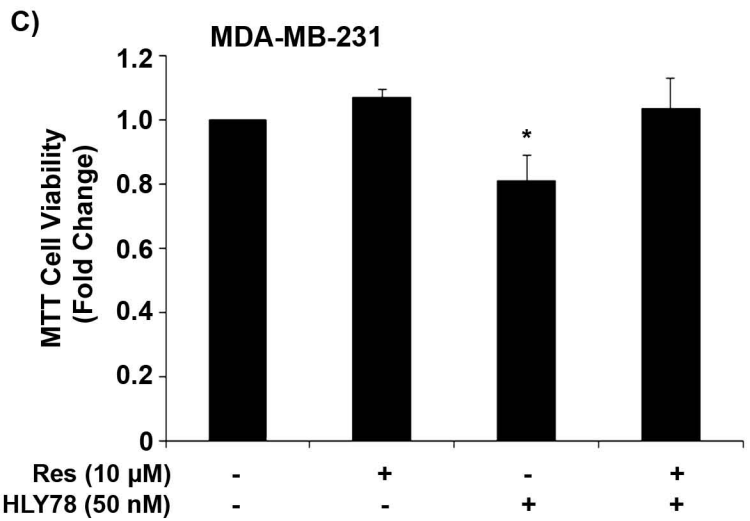


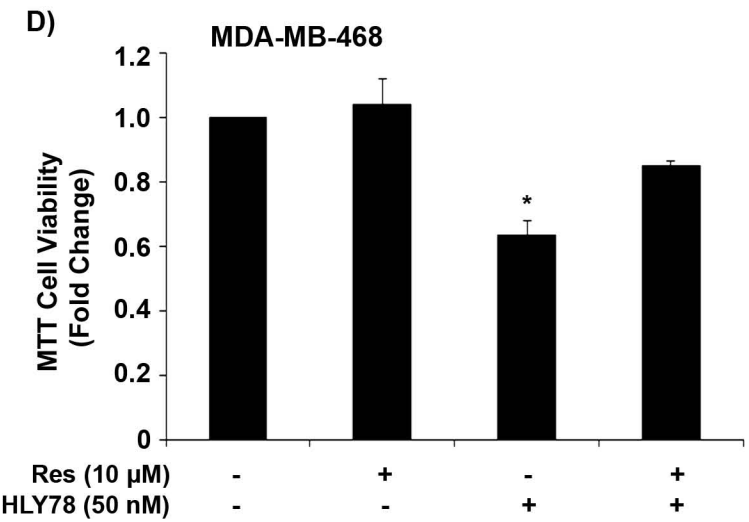
E)

Cell lines	Bliss values	Net effect
MCF-7	1.15 ± 0.05	Synergy
BT474	1.23 ± 0.05	Synergy
MDA-MB-231	1.04 ± 0.02	Additive
MDA-MB-468	0.88 ± 0.03	Antagonism





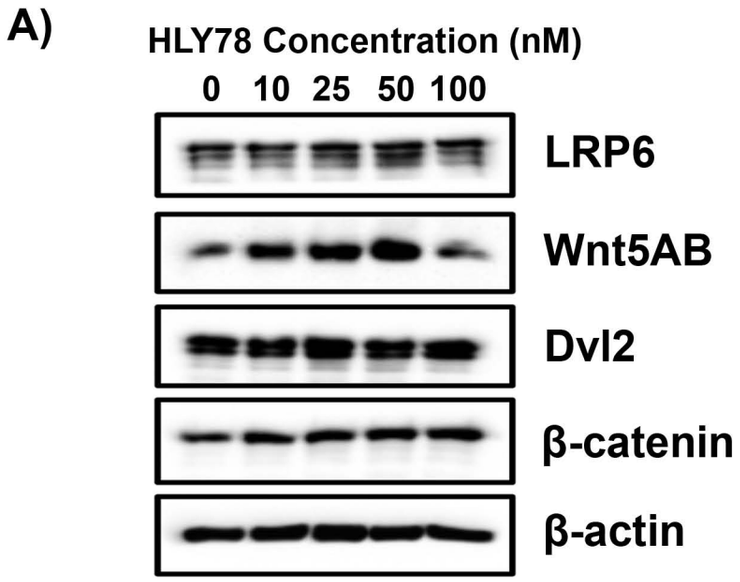


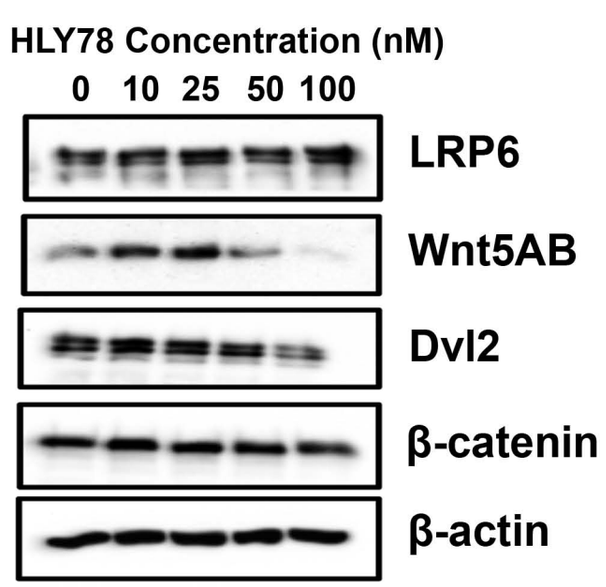


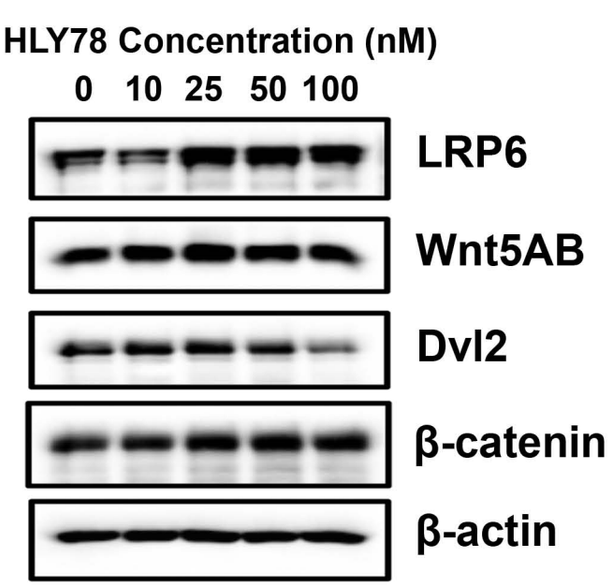
E)

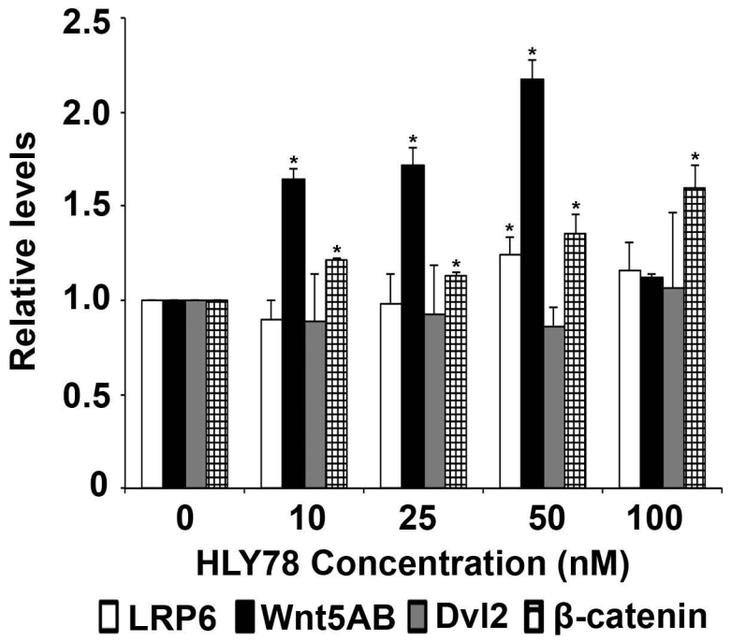
Cell lines	Bliss values	Net effect
MCF-7	0.90 ± 0.05	Antagonism
BT474	0.92 ± 0.02	Antagonism
MDA-MB-231	0.84 ± 0.04	Antagonism
MDA-MB-468	0.74 ± 0.05	Antagonism

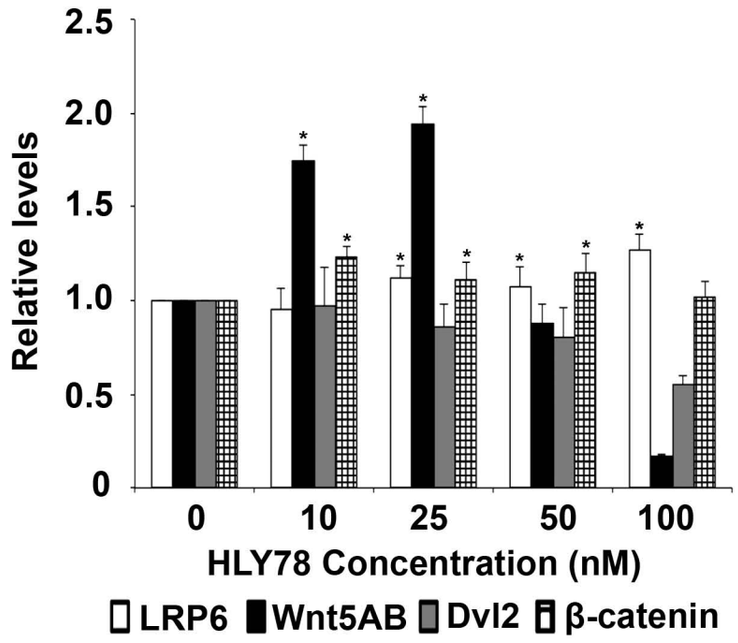
A)

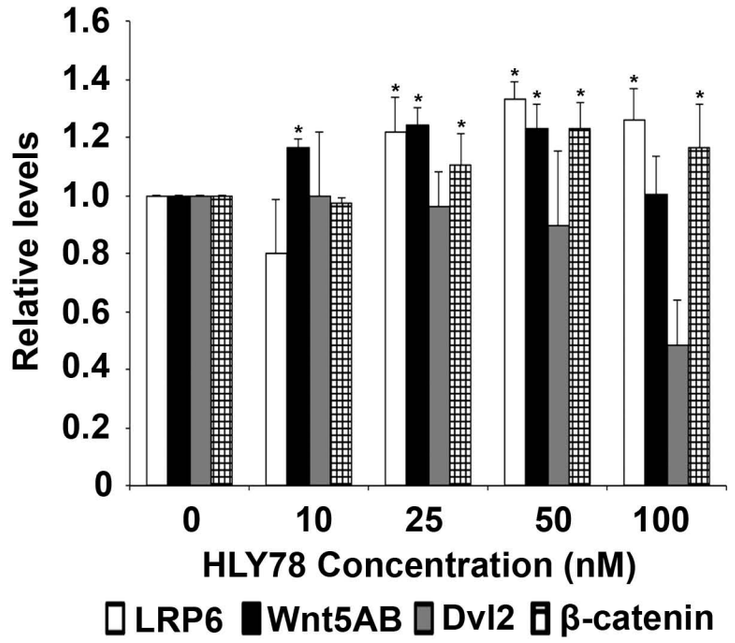

HLY78 Concentration (nM)
0 10 25 50 100
LRP6
Wnt5AB
Dvl2
β-catenin
β-actin
MCF-7


HLY78 Concentration (nM)
0 10 25 50 100
LRP6
Wnt5AB
Dvl2
β-catenin
β-actin
MDA-MB-231

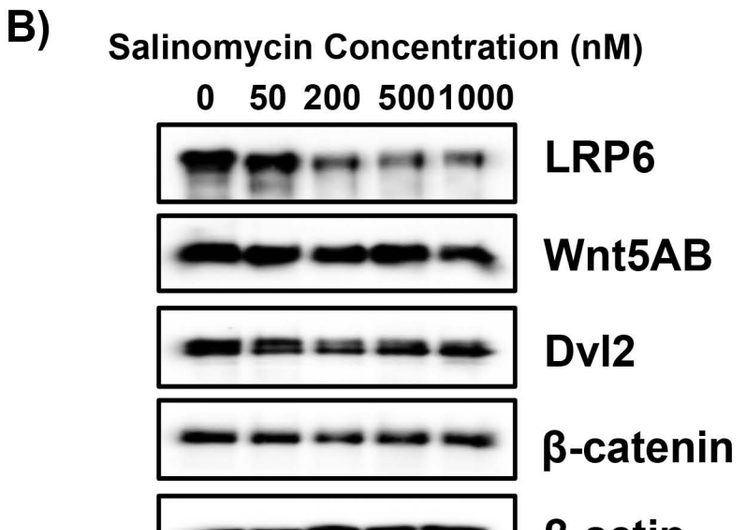

HLY78 Concentration (nM)
0 10 25 50 100
LRP6
Wnt5AB
Dvl2
β-catenin
β-actin
MDA-MB-468

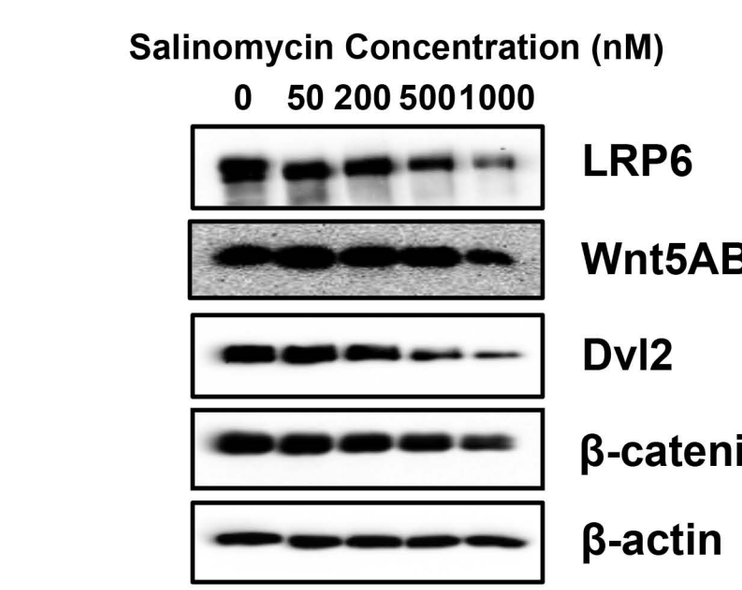

Relative levels
HLY78 Concentration (nM)
0 10 25 50 100
LRP6 Wnt5AB Dvl2 β-catenin


Relative levels
HLY78 Concentration (nM)
0 10 25 50 100
LRP6 Wnt5AB Dvl2 β-catenin


Relative levels
HLY78 Concentration (nM)
0 10 25 50 100
LRP6 Wnt5AB Dvl2 β-catenin

B)


Salinomycin Concentration (nM)
0 50 200 500 1000
LRP6
Wnt5AB
Dvl2
β-catenin
β-actin
MCF-7


Salinomycin Concentration (nM)
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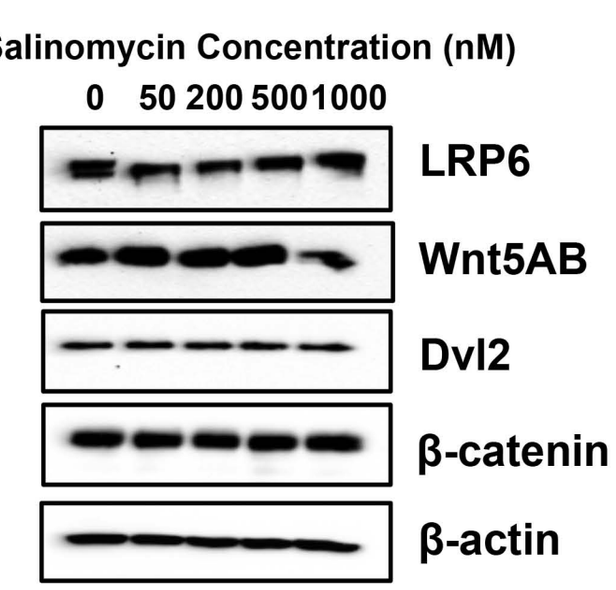
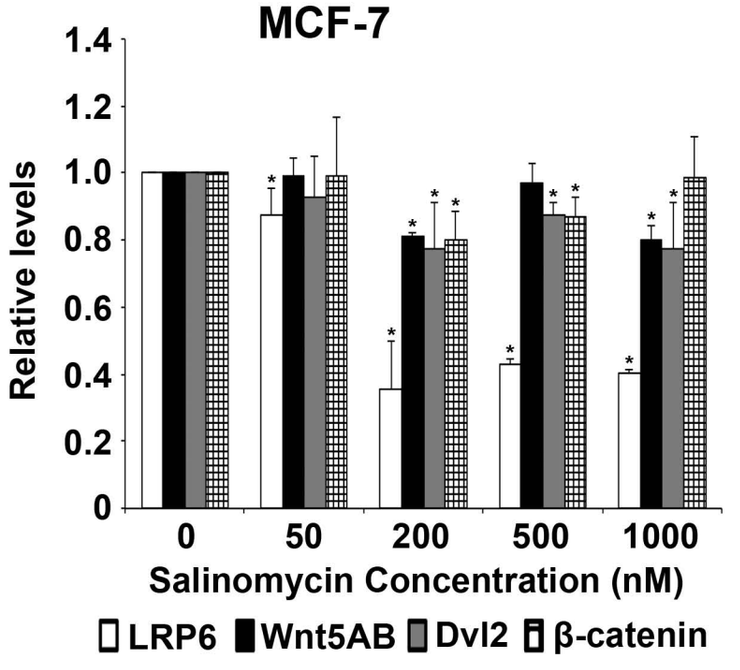
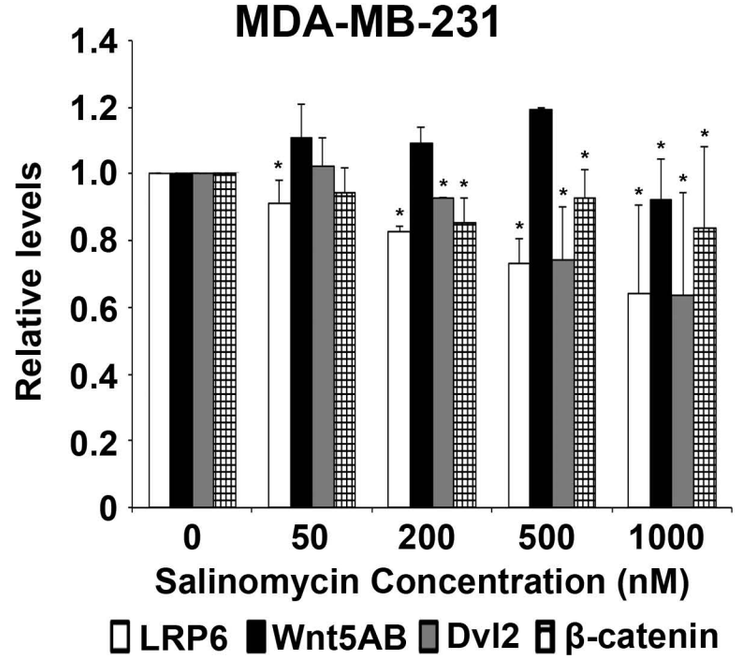
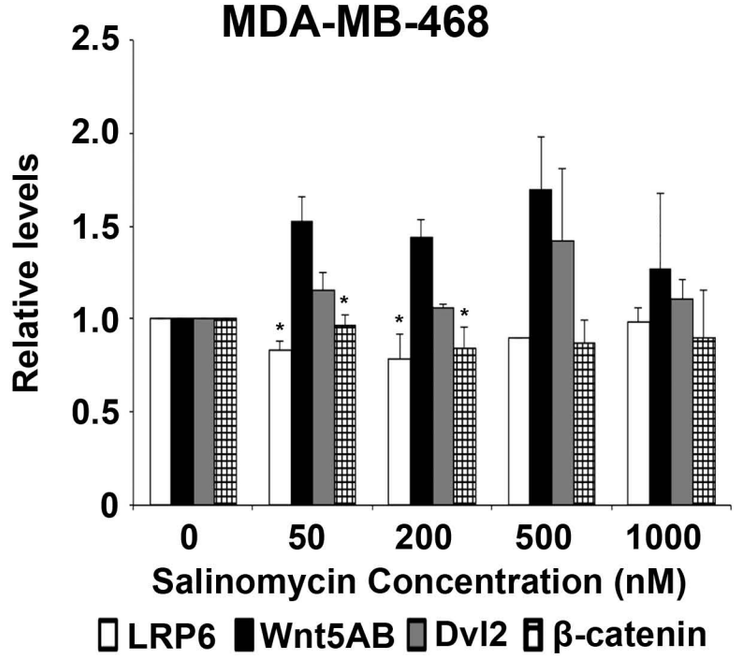
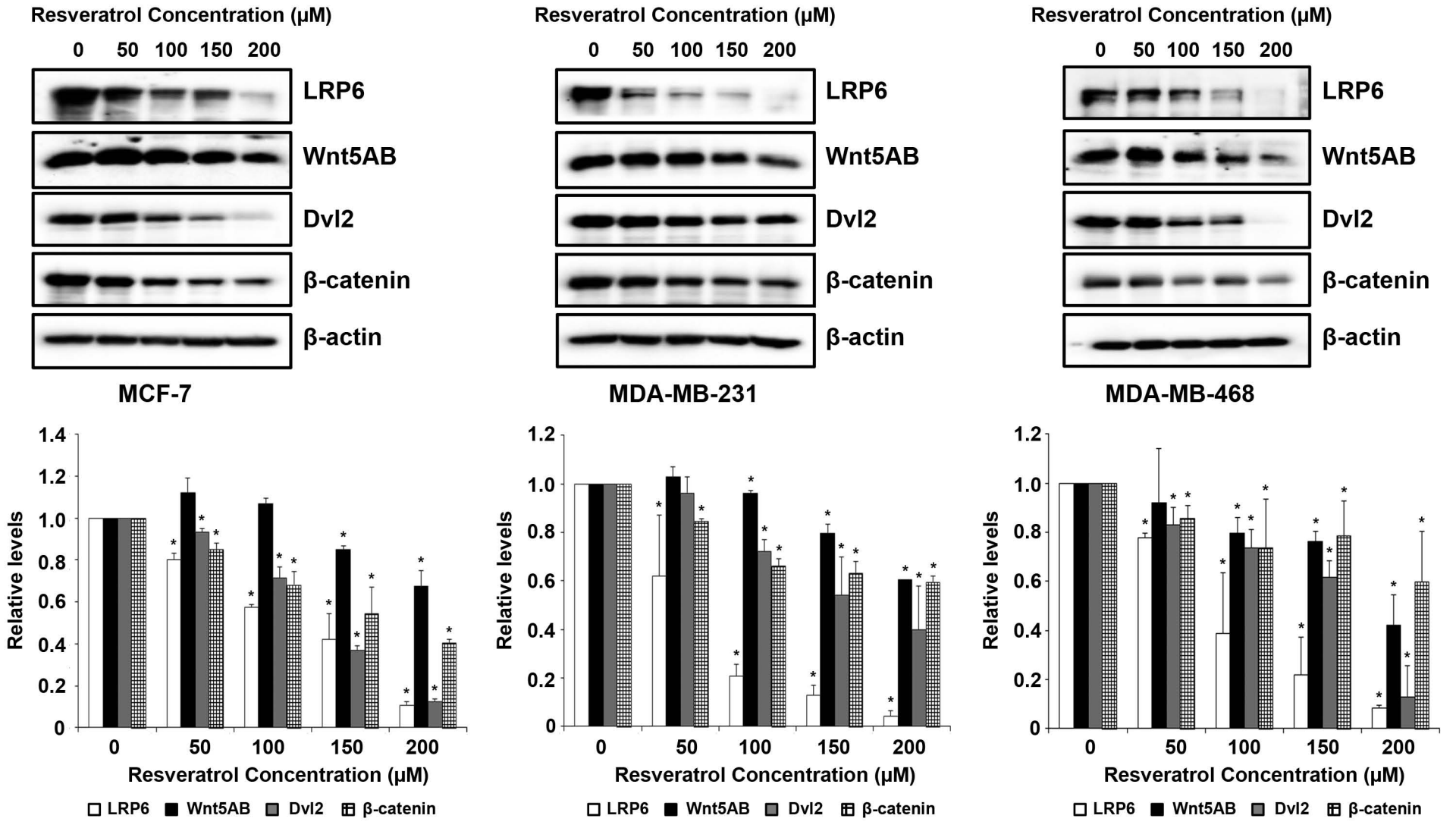
LRP6
Wnt5AB
Dvl2
β-catenin
β-actin
MDA-MB-231


Salinomycin Concentration (nM)
0 50 200 500 1000
LRP6
Wnt5AB
Dvl2
β-catenin
β-actin
MDA-MB-468


Relative levels
Salinomycin Concentration (nM)
0 50 200 500 1000
LRP6 Wnt5AB Dvl2 β-catenin


Relative levels
Salinomycin Concentration (nM)
0 50 200 500 1000
LRP6 Wnt5AB Dvl2 β-catenin


Relative levels
Salinomycin Concentration (nM)
0 50 200 500 1000
LRP6 Wnt5AB Dvl2 β-catenin


Resveratrol Concentration (μM)
0 50 100 150 200
LRP6
Wnt5AB
Dvl2
β-catenin
β-actin
MCF-7
MDA-MB-231
MDA-MB-468
Relative levels
Resveratrol Concentration (μM)
LRP6 Wnt5AB Dvl2 β-catenin