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Salinomycin Suppresses LRP6 Expression and Inhibits Both Wnt/β-catenin and mTORC1 Signaling in Breast and Prostate Cancer Cells

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ABSTRACT

Emerging evidence indicates that activation of Wnt/β-catenin signaling at the cell surface results in inhibition of glycogen synthase kinase 3β (GSK3β), leading to activation of mTORC1 signaling in cancer cells. The low density lipoprotein receptor-related protein-6 (LRP6) is an essential Wnt co-receptor for Wnt/β-catenin signaling. Salinomycin is a novel small molecule inhibitor of LRP6. In the present study, we found that LRP6 overexpression induced mTORC1 signaling activation in cancer cells, and that salinomycin was not only a potent Wnt/β-catenin signaling inhibitor, but also a strong mTORC1 signaling antagonist in breast and prostate cancer cells. Mechanistically, salinomycin activated GSK3β in cancer cells. Moreover, salinomycin was able to suppress the expression of cyclin D1 and survivin, two targets of both Wnt/β-catenin and mTORC1 signaling, in prostate and breast cancer cells, and displayed remarkable anticancer activity. Our results present novel mechanisms underlying salinomycin-mediated cancer cell death. J. Cell. Biochem. 115: 1799–1807, 2014.

KEY WORDS: SALINOMYCIN; LRP6; Wnt SIGNALING; mTORC1 SIGNALING; CANCER

The low-density lipoprotein receptor-related protein-6 (LRP6) is a member of the low density lipoprotein receptor family, and acts as a co-receptor for Wnt ligands, which interact with both the seven transmembrane receptor of the Frizzled (Fzd) family and LRP6 to activate the Wnt/β-catenin signaling pathway. Wnt/β-catenin signaling is important for cancer progression, including tumor initiation, tumor growth, cell senescence, cell death, differentiation, and metastasis [Clevers and Nusse, 2012; Polakis, 2012; Anastas and Moon 2013]. LRP6 is upregulated in several types of malignant tissues [Lindvall et al., 2009; Liu et al., 2010, 2012b; Yang et al., 2011; Tung et al., 2012]. Recent studies have demonstrated that the Wnt/β-catenin signaling pathway is preferentially activated in human triple negative breast cancer (TNBC), and that LRP6 is upregulated in this subtype of human breast cancer [Lindvall et al., 2009; Liu et al., 2010; Yang et al., 2011]. LRP6 expression is also significantly upregulated in prostate patients with metastatic disease compared to those without metastasis, and is associated with a significantly increased risk of recurrent disease [Liu et al., 2012b]. As an essential Wnt co-receptor to activate Wnt/β-catenin signaling, LRP6 is a potential therapeutic target for the development of novel anticancer drugs [King et al., 2012].

Mammalian target of rapamycin (mTOR) is a protein kinase ubiquitously expressed within cells and a validated target in the treatment of cancer. The rapamycin-sensitive mTOR complex (mTORC1; containing raptor) activates protein synthesis through modulation of the 40S ribosomal protein S6 kinase (S6K) and the translational initiation factor eIF-4E binding protein 1 (4E-BP1). Being downstream of AKT, mTORC1 has been described as the most essential effector in driving cell proliferation and susceptibility to oncogenic transformation [Guertin and Sabatini, 2007; Garcia and Danielpour, 2008; Dancey, 2010].

There is a crosstalk between the Wnt/β-catenin and mTORC1 signaling pathways, and accumulating evidence indicates that activation of Wnt/β-catenin signaling at the cell surface results in

Abbreviations: CM 4E-BP1, the translational initiation factor eIF-4E binding protein 1; CM, conditioned medium; CSCs, cancer stem cells; Dkk1, Dickkopf-1; FBS, fetal bovine serum; Fzd, Frizzled; GSK3β, glycogen synthase kinase 3β; LRP6, low-density lipoprotein receptor-related protein-6; mTOR, mammalian target of rapamycin; mTORC1, the rapamycin-sensitive mTOR complex; S6K, the 40S ribosomal protein S6 kinase; TNBC, triple negative breast cancer.

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upregulation of mTORC1 signaling in cancer cells [Inoki et al., 2006; Fujishita et al., 2008; Castilho et al., 2009; Huang et al., 2009, 2012 Gao et al., 2010; Hagenmueller et al., 2010; Liu et al., 2012a; Kwan et al., 2013; Tahir et al., 2013; Valvezan et al., 2014]. It has been demonstrated that glycogen synthase kinase 3β (GSK3β) regulated both Wnt/β-catenin and mTORC1 signaling in mouse hematopoietic stem cells [Huang et al., 2009, 2012], and that Wnt proteins activated mTORC1 signaling by a mechanism that involves the inhibition of GSK3β [Inoki et al., 2006]. Furthermore, it was found that LRP6$^{+/-}$ mice displayed an impaired activity of mTORC1 pathway in brown adipose tissue [Liu et al., 2012a], and that knockdown of LRP6 suppressed mTORC1 signaling in prostate cancer cells [Tahir et al., 2013]. These studies indicate that LRP6 is associated with Wnt protein-induced activation of mTORC1signaling in cancer cells.

Current cancer treatments, such as chemotherapy, hormone therapy, and radiotherapy, are successful at destroying bulk cancer cells but fail to eliminate cancer stem cells (CSCs). CSCs are characterized by tumorigenic properties and the ability to self-renew, form differentiated progeny, and develop resistance to therapy. The Wnt/β-catenin signaling pathway has been implicated in the control over various types of stem cells and may act as a niche factor to maintain CSCs in a self-renewing state [Clevers and Nusse, 2012; Polakis 2012; Anastas and Moon, 2013]. Salinomycin, a polyether ionophore antibiotic isolated from *Streptomyces albus*, is used as an antibiotic in animal husbandry [Miyazaki et al., 1974]. Gupta et al. developed and implemented a high throughput screening method to identify agents with specific toxicity for epithelial CSCs, and identified salinomycin as a selective inhibitor of breast CSCs [Gupta et al., 2009]. Promising results from preclinical trials in human xenograft mice and a few clinical pilot studies reveal that salinomycin could be a promising novel anticancer agent despite its largely unknown mechanism of action [Huczynski, 2012; Naujokat and Steinhart, 2012; Zhou et al., 2013]. It was recently reported that salinomycin is a Wnt/β-catenin signaling inhibitor through induction of Wnt co-receptor LRP6 degradation in chronic lymphocytic leukemia cells [Lu et al., 2011a]. In the present study, we demonstrated that salinomycin is not only a potent Wnt/β-catenin signaling inhibitor, but also a strong mTORC1 signaling antagonist in breast and prostate cancer cells, revealing novel mechanisms underlying salinomycin-mediated cancer cell death.

MATERIALS AND METHODS

MATERIALS

Salinomycin was purchased from Sigma, and dissolved as 10 mM stock in DMSO. Plasmid pCS-Myc-hLRP6 containing the full-length human LRP6 cDNA was provided by Dr. Christof Niehrs (Deutsches Krebsforschungszentrum, Heidelberg, Germany), and plasmid pGST-E-cadherin was provided by Dr. Gail Johnson (University of Rochester). The Super8XTOPFlash luciferase construct was provided by Dr. Randall T. Moon (University of Washington, Seattle). A β-galactosidase-expressing vector was from Promega. Polyclonal anti-LRP6 (C-10) (sc-25317) and monoclonal anti-survivin (D-8) (sc-17779) were from Santa Cruz Biotechnology. Monoclonal anti-axin2 (76G2) (#2151), anti-S6 (5G10) (#2217), anti-phospho-S6 (91B2) (#4857), anti-GSK3β (27C10) (#9315), anti-phospho-GSK3β (D3A4) (#9322) and polyclonal anti-phospho-LRP6 (ser1490) (#2568), anti-p70S6K (#9256), anti-phospho-p70S6K (#9205) were purchased from Cell Signaling Technology. Polyclonal rabbit anti-cyclin D1 (#04-221) was from EMD Millipore. Monoclonal anti-β-catenin (#610154) was from BD Biosciences. Monoclonal anti-actin (A2228) was from Sigma. Peroxidase labeled anti-mouse antibody and ECL system were purchased from Amersham Life Science. The luciferase and β-galactosidase assay systems were from Promega. Tissue culture media, fetal bovine serum (FBS), and plastic-ware were obtained from Life Technologies, Inc. Proteinase inhibitor cocktail Complete™ was obtained from Boehringer Mannheim.

CELL CULTURE AND CONDITIONED MEDIA

All cell lines were obtained from ATCC and grown under standard cell culture conditions at 37 °C in a humidified atmosphere with 5% CO_2. LRP6-transduced MCF-7 cells and the corresponding control cells were prepared as described before [Li et al., 2004], and cultured in DMEM medium containing 10% of fetal bovine serum (FBS), 2 mM of L-glutamine, 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 350 mg/mL of G418. HEK293 cells and breast cancer MCF-7, HS578T, and MDA-MB-231 cells were cultured in the same above medium without G418. The prostate cancer PC-3 and DU145 cells were cultured in RPMI-1640 medium containing 10% FBS, 2 mM of L-glutamine, 100 units/mL of penicillin, and 100 μg/mL of streptomycin.

Wnt3A-conditioned medium (CM) and parental L cell control CM were prepared according to manufacturer's specifications. Briefly, L Wnt3A cells or parental L cells were cultured in 15-cm dishes with 30 ml culture medium. After the cells reached confluence, the medium was replaced with fresh complete medium without G418. After 3 days of incubation, the Wnt3A CM and L cell control CM were collected, filtered, and stored at −80 °C.

WESTERN BLOTTING

Cells in six-well plates were lysed in 0.5 mL of lysis buffer (phosphate-buffered saline containing 1% Triton X-100 and 1 mM PMSF) at 4 °C for 10 min. Equal quantities of protein were subjected to SDS-PAGE under reducing conditions. Following transfer to immobilon-P transfer membrane, successive incubations with a primary antibody, and a horseradish peroxidase-conjugated secondary antibody were carried out for 60–120 min at room temperature. The immunoreactive proteins were then detected using the ECL system. Films showing immunoreactive bands were scanned by Hp Scanjet 5590.

CYTOSOLIC FREE β-CATENIN ANALYSIS WITH GST-E-CADHERIN BINDING ASSAY

The GST-E-cadherin binding assay was carried out exactly as previously described [Lu et al., 2008]. Uncomplexed cytosolic free β-catenin present in 100 μg of total cell lysate was subjected to SDS-PAGE and detected using the monoclonal antibody to β-catenin.

APOPTOSIS EVALUATION

Apoptosis was assessed exactly as previously described with cell death detection ELISA kit purchased from Roche Diagnostics [Lu

et al., 2011b]. Briefly, cells were cultured in T25 flasks for the desired duration. 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 flasks with the cells being lysed using the buffer as described above for Western blotting.

LUCIFERASE REPORTER ASSAY FOR WNT-β-CATENIN SIGNALING

Cancer cells were plated into 24-well plates. After overnight culture, the cells were transiently transfected with the Super8XTOPFlash luciferase construct and β-galactosidase-expressing vector along with LRP6 plasmid along with or without LRP6 plasmid. After 24 h incubation, cells were treated with salinomycin at the indicated concentrations. Cells were then lysed 24 h later and both luciferase and β-galactosidase activities were determined. The luciferase activity was normalized to the β-galactosidase activity.

CELL VIABILITY ASSAY

Cancer cells were seeded into 96-well tissue culture treated microtiter plates at a density of 5000 cells/well. RPMI-1640 containing 10% FBS was used as assay media for PC-3 and DU145 cells, while DMEM containing 10% FBS was used as assay media for HS578T and MDA-MB-231 cells. After 24 h incubation, the cells were treated with salinomycin at the indicated concentrations for 72 h. Cell viability was measured by the Cell Titer Glo Assay, which is a luminescent assay that is an indicator of live cells as a function of metabolic activity and ATP content.

COLONY FORMATION ASSAY

Cancer cells were seeded at a density of 500 cells/well into six-well plates. Sixteen hours after the plates had been set up, Salinomycin was added at a concentration of 0.25 μM or 1 μM, and media were replenished every 3 days. After being incubated for 10–14 days, colonies were fixed with 4% formaldehyde, stained with 0.5 mg/mL crystal violet, and imaged on a FluorChem HD2 Imager System (Alpha Innotech).

STATISTICS

Statistical analyses were performed using Student's unpaired *t*-test. Data were presented as mean ± SD. Differences at $P < 0.05$ were considered statistically significant.

RESULTS

SALINOMYCIN INHIBITS LRP6 EXPRESSION AND WNT/β-CATENIN SIGNALING IN BREAST AND PROSTATE CANCER CELLS

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. It has been reported that salinomycin reduced LRP6 expression and phosphorylation and inhibited Wnt/β-catenin signaling in HEK293 cells and chronic lymphocytic leukemia cells [Lu et al., 2011a]. We confirmed that salinomycin inhibited LRP6- and Wnt3A-induced Wnt/β-catenin signaling in a dose-dependent manner in HEK293 cells (Fig. 1). Importantly, salinomycin was able to significantly inhibit Wnt/β-catenin signaling at a low concentration of 0.25 μM, indicating that salinomycin is a potent inhibitor of the Wnt/β-catenin signaling pathway.

To determine whether salinomycin blocks Wnt/β-catenin signaling in other types of cancer, we examined the levels of LRP6 expression and phosphorylation in breast cancer HS578T and MDA-MB-231 cells and prostate cancer PC-3 and DU145 cells. We found that the levels of LRP6 expression and phosphorylation in breast and prostate cancer cells were significantly reduced after salinomycin treatment (Fig. 2). Uncomplexed cytosolic β-catenin (free β-catenin) can translocate to the cell nucleus and bind transcription factors, such as the T-cell factor or lymphocyte enhancer factor, leading to the transcription of Wnt target genes. We also found that cytosolic free β-catenin levels in breast and prostate cancer cells were significantly reduced after salinomycin treatment (Fig. 2). Furthermore, expression of axin2, a specific transcriptional target of the Wnt/β-catenin signaling pathway, was significantly deceased after salinomycin treatment in breast and prostate cancer cells (Fig. 2).

LRP6 OVEREXPRESSION ENHANCES mTORC1 SIGNALING IN CANCER CELLS

Inhibition of Wnt/β-catenin at the cell surface can result in downregulation of mTORC1 signaling in cancer cells [Inoki et al., 2006; Huang et al., 2009, 2012 Liu et al., 2012a; Tahir et al., 2013]. It was reported that LRP6 deficiency caused inhibition of mTORC1 signaling in prostate cancer PC-3 cells [Tahir et al., 2013]. Furthermore, Wnt ligands were able to stimulate mTORC1 signaling both in vitro and in vivo, while LRP6 antagonist Dickkopf-1 (Dkk1) inhibited Wnt3A-induced mTORC1 signaling in vitro [Inoki et al., 2006]. To further test whether LRP6 plays a role in mTORC1 signaling, we examined the effect of LRP6 overexpression on mTORC1 signaling in cancer cells. We found that breast cancer MCF-7 cells stably transduced with LRP6 displayed higher levels of p70S6K phosphorylation and S6 phosphorylation than the corresponding control MCF-7 cells (Fig. 3).

SALINOMYCIN INHIBITS mTORC1 SIGNALING IN BREAST AND PROSTATE CANCER CELLS

As salinomycin suppressed LRP6 expression and phosphorylation in breast and prostate cancer cells, we then tested whether salinomycin inhibits mTORC1 signaling in cancer cells too. As shown in Figure 4, salinomycin at concentrations of 0.5–4 μM was able to significantly suppress phosphorylation of p70S6K and S6 in breast cancer HS578T and MDA-MB-231 cells and prostate cancer PC-3 and DU145 cells, indicating that salinomycin is a potent of mTORC1 inhibitor in breast and prostate cancer cells.

It has been found that GSK3β suppresses mTORC1 signaling [Inoki et al., 2006; Huang et al., 2009, 2012], and that Wnt proteins activate mTORC1 signaling by a mechanism that involves the inhibition of GSK3β [Inoki et al., 2006]. As shown in Figure 5, salinomycin was able to enhance GSK3β phosphorylation in breast cancer MDA-MB-231 cells and prostate cancer DU145 cells, indicating that GSK3β activation is associated with salinomycin-induced inhibition of mTORC1signaling in breast and prostate cancer cells.

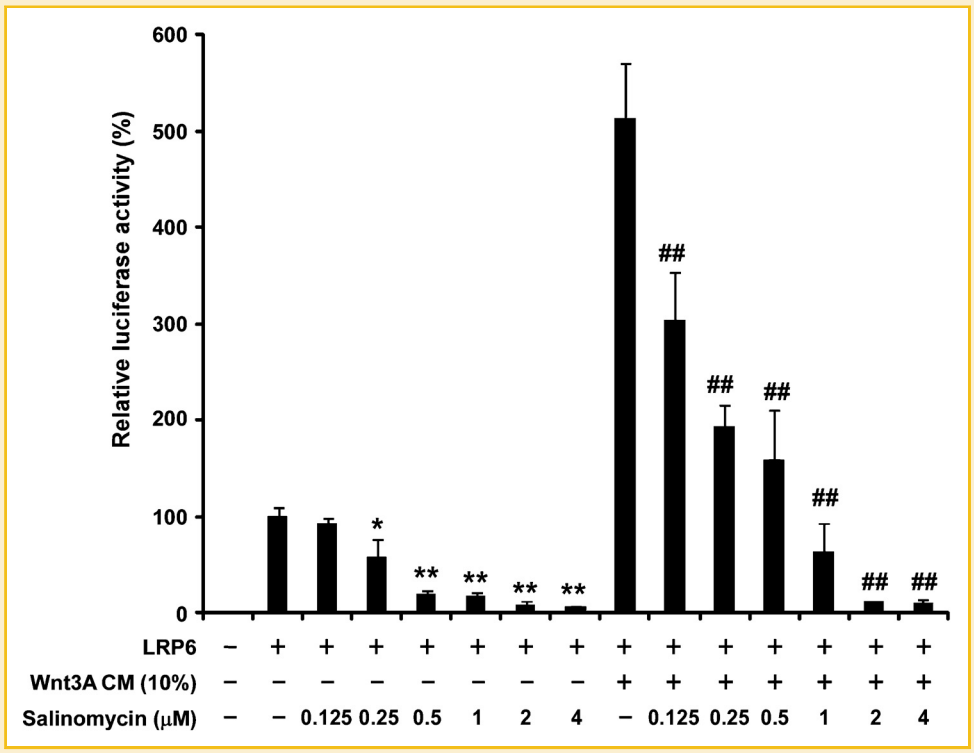

Fig. 1. Salinomycin inhibits LRP6-induced Wnt/β signaling in HEK293 cells. HEK293 cells in 24-well plates were transiently transfected with LRP6 plasmid or the corresponding control vector, along with Super8XTOPFlash construct and β-galactosidase-expressing vector in each well. After being incubated for 24 h, cells were treated with salinomycin at indicated concentrations in the absence or presence of Wnt3A CM (10%) for 24 h. The luciferase activity was then measured 24 h later with normalization to the activity of the β-galactosidase. Values are averages of three determinations with the standard deviations indicated by error bars. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to cells transfected with LRP6. $^{\#\#}P < 0.01$ compared to cells transfected with LRP6 and treated with Wnt3A CM.

SALINOMYCIN INHIBITS CYCLIN D1 AND SURVIVIN EXPRESSION IN BREAST AND PROSTATE CANCER CELLS

Cyclin D1 is critical for cancer cell proliferation, and is a transcriptional target of both Wnt/β-catenin signaling [Shtutman et al., 1999; Tetsu and McCormick, 1999] and mTORC1 signaling [Averous et al., 2008]. Survivin is a dual regulator of cancer cell proliferation and cell death, and its expression is also regulated by both Wnt/β-catenin signaling [Kim et al., 2003; Ma et al., 2005] and mTORC1 signaling [Vaira et al., 2007]. As expected, salinomycin treatment resulted in downregulation of cyclin D1 and survivin expression in both breast and prostate cancer cells (Figure 6).

SALINOMYCIN INHIBITS BREAST AND PROSTATE CANCER CELL GROWTH AND INDUCES CANCER CELL APOPTOSIS

Given that salinomycin blocked Wnt/β-catenin and mTORC1-signaling and inhibited cyclin D1 and survivin expression in cancer cells, we then examined the effect of salinomycin on cancer cell proliferation and apoptosis. As shown in Figure 7A, salinomycin inhibited cancer cell viability in a concentration-dependent manner in breast cancer HS578T and MDA-MB-231 cells and prostate cancer PC-3 and DU145 cells. Notably, salinomycin was able to significantly suppress cancer cell growth at a concentration as low as 80 nM. Furthermore, exposure to salinomycin at 2 μM for 24 h significantly induced apoptotic DNA fragmentation in cancer cells (Fig. 7B). Finally, we performed the colony formation assay, and found that salinomycin at 0.25 and

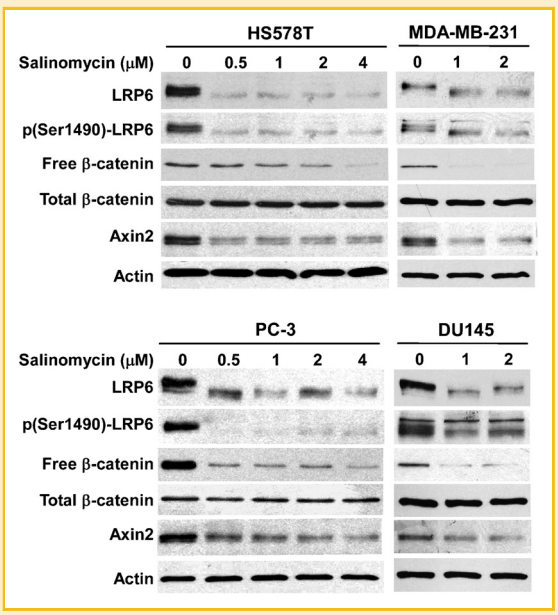

Fig. 2. Salinomycin suppresses Wnt/β-catenin signaling in cancer cells. Breast cancer HS578T and MDA-MB-231 cells and prostate cancer PC-3 and DU145 cells in 6-well plates were treated with salinomycin at the indicated concentrations for 24 h. The levels of cytosolic free β-catenin, total cellular β-catenin, LRP6, phospho-LRP6 and axin2 were examined by Western blotting. All the samples were also probed with anti-actin antibody to verify equal loading.

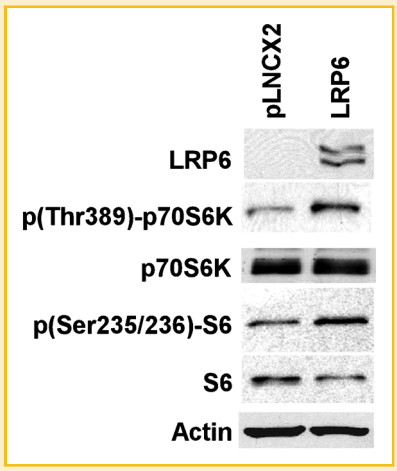

Fig. 3. LRP6 overexpression enhances mTORC1 signaling. MCF-7 cells stably transduced with LRP6 and the corresponding control cells were cultured in 6-well plates. The levels of LRP6, p70S6K, phospho-p70S6K, S6 and phospho-S6 were examined by Western blotting. All the samples were also probed with anti-actin antibody to verify equal loading.

1 μM significantly suppressed colony formation in both breast and prostate cancer cells (Fig. 8).

DISCUSSION

Emerging data indicate that both the Wnt/β-catenin and mTORC1 signaling pathways are play critical roles in cancer development and progression [Guertin and Sabatini, 2007; Garcia and Danielpour, 2008; Dancey, 2010; Clevers and Nusse, 2012; Polakis, 2012; Anastas and Moon 2013]. Therefore, a small molecule that successfully inhibits both Wnt/β-catenin and mTORC1 signaling might provide an attractive agent for chemotherapeutic use. Salinomycin, a broad spectrum antibiotic and a coccidiostat, has recently been shown to induce apoptosis and cause growth inhibition in diverse types of apoptosis- and chemotherapeutic-resistant cancer cells, and may be considered as a promising novel anticancer agent despite its largely unknown mechanism of action [Huczynski, 2012; Naujokat and Steinhart, 2012; Zhou et al., 2013]. Herein, we demonstrated that salinomycin inhibited both the Wnt/β-catenin and mTORC1 signaling pathways in breast and prostate cancer cells, providing novel mechanisms underlying salinomycin-mediated anticancer activity.

As an essential Wnt-co-receptor, LRP6 is overexpressed in several types of cancer [Lindvall et al., 2009; Liu et al., 2010; Yang et al., 2011; Tung et al., 2012; Liu et al., 2012b], and represents a promising therapeutic target for the development of novel anticancer drugs [King et al., 2012]. It has been demonstrated that

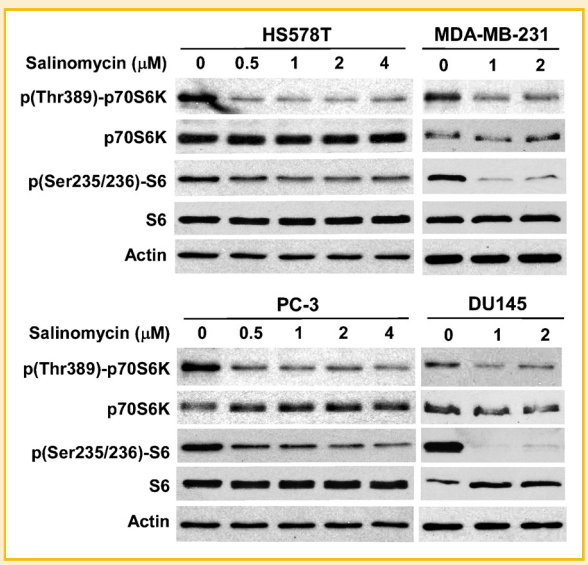

Fig. 4. Salinomycin suppresses mTORC1 signaling in cancer cells. Breast cancer HS578T and MDA-MB-231 cells and prostate cancer PC-3 and DU145 cells in six-well plates were treated with salinomycin at the indicated concentrations for 24 h. The levels of total cellular p70S6K, phospho-p70S6K, S6 and phospho-S6 were examined by Western blotting. All the samples were also probed with anti-actin antibody to verify equal loading.

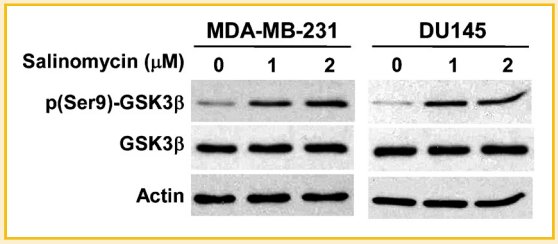

Fig. 5. Salinomycin enhances GSK3β phosphorylation in cancer cells. Breast cancer MDA-MB-231 cells and prostate cancer DU145 cells in 6-well plates were treated with salinomycin at the indicated concentrations for 24 h. The levels of total cellular GSK3β and phospho-GSK3β were examined by Western blotting. All the samples were also probed with anti-actin antibody to verify equal loading.

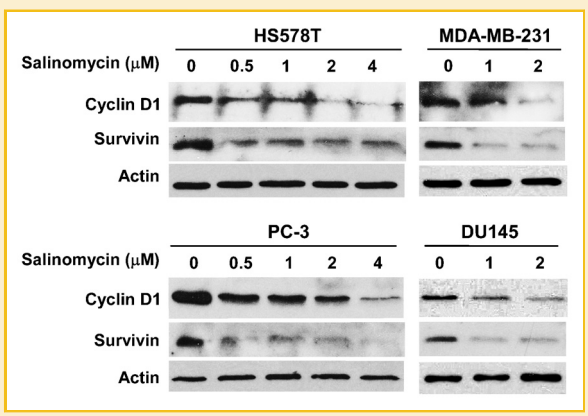

Fig. 6. Salinomycin inhibits cyclin D1 and survivin expression in cancer cells. Breast cancer HS578T and MDA-MB-231 cells and prostate cancer PC-3 and DU145 cells in six-well plates were treated with salinomycin at the indicated concentrations for 48 h. The levels of cyclin D1 and survivin were examined. All the samples were also probed with anti-actin antibody to verify equal loading.

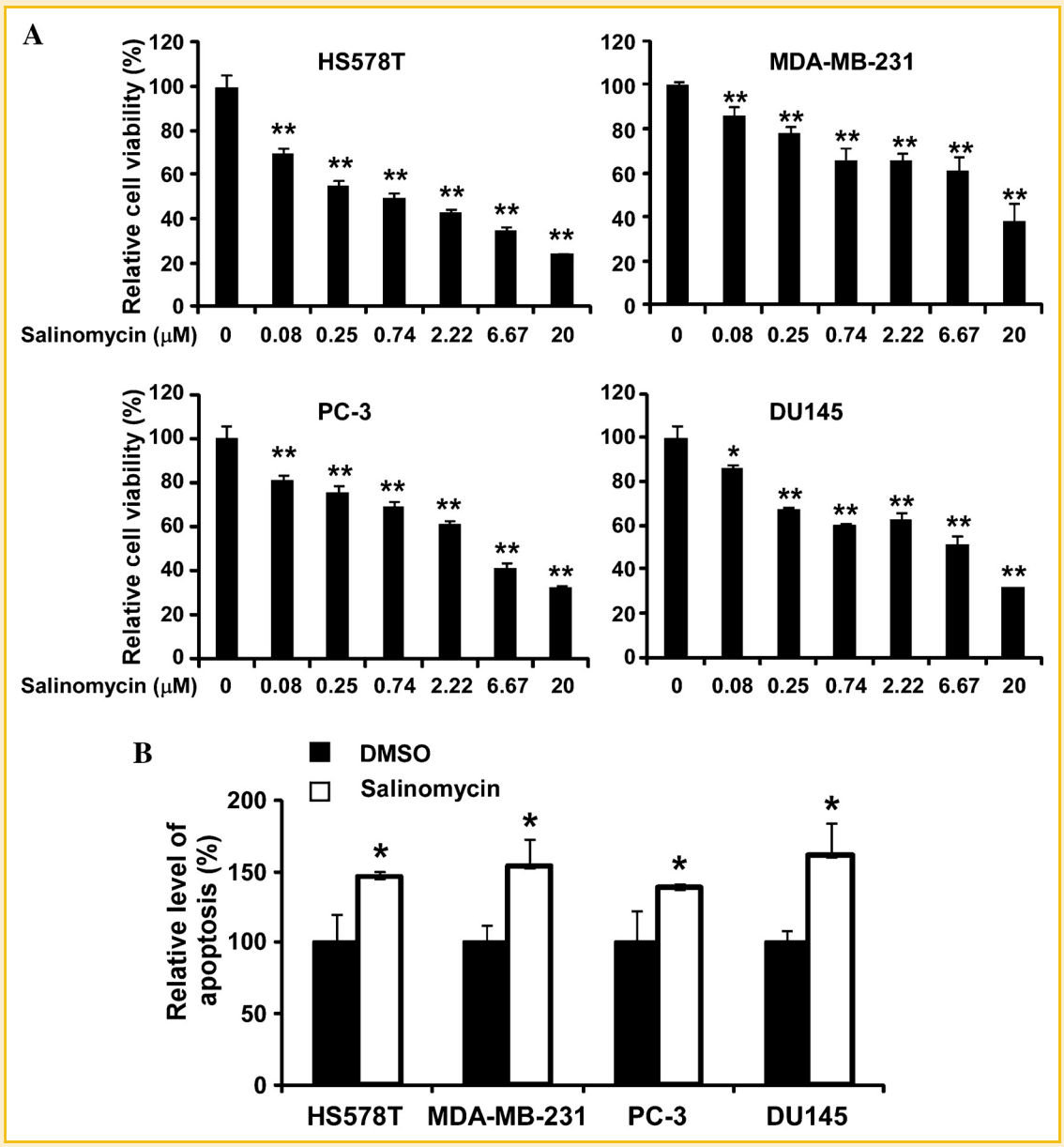

Fig. 7. Effects of salinomycin on cancer cell proliferation and apoptosis. (A) Breast cancer HS578T and MDA-MB-231 cells and prostate cancer PC-3 and DU145 cells in 96-well plates were treated with salinomycin for 72 h. Cell viability was measured by the Cell Titer Glo Assay system. (B) Cancer cells were treated with salinomycin (2 μM) for 24 h. Floating and attached cells were combined for apoptosis detection by the Cell Death ELISA kit from Roche Diagnostics as described in Materials and Methods. All the values are the average of triplicate determinations with the s.d. indicated by error bars. *$P < 0.05$, **$P < 0.01$ compared to cells treated with DMSO.

LRP6 deficiency in human TNBC MDA-MB-231 cells resulted in significantly decreases of Wnt/β-catenin signaling, cell proliferation, and tumor growth in vivo [Liu et al., 2010], and that blocking Wnt/β-catenin signaling by N-myc downstream regulated gene-1 (NDRG1), a tumor metastasis suppressor which interacts with LRP6 and represses Wnt/β-catenin signaling, led to drastic suppression of metastatic phenotypes of mammary tumor cells in vitro and in vivo [Liu et al., 2012b]. In addition, small molecule LRP6 inhibitors were able to inhibit human breast and prostate cancer cell proliferation [Lu et al., 2011b, 2012, 2014], and LRP6 antibodies antagonized Wnt1- and Wnt3-induced Wnt/β-catenin signaling and suppressed the growth of allografted tumor cells derived from MMTV-Wnt1 and MMTV-Wnt3 tumors [Ettenberg et al., 2010; Gong et al., 2010]. Moreover, the recombinant Mesd protein and its C-terminal region peptide, two universal inhibitors of LRP6, markedly inhibited Wnt/β-catenin signaling in prostate and breast cancer cells, leading to inhibition of cancer cell proliferation in vitro and tumor growth in vivo [Liu et al., 2010; Lu et al., 2010; Lin et al., 2011, 2013]. In the present study, we demonstrated that salinomycin is a potent inhibitor of Wnt/β-catenin signaling by suppressing LRP6 expression in prostate and breast cancer cells. We also found that the effect of salinomycin on LRP6 expression occurred at concentrations

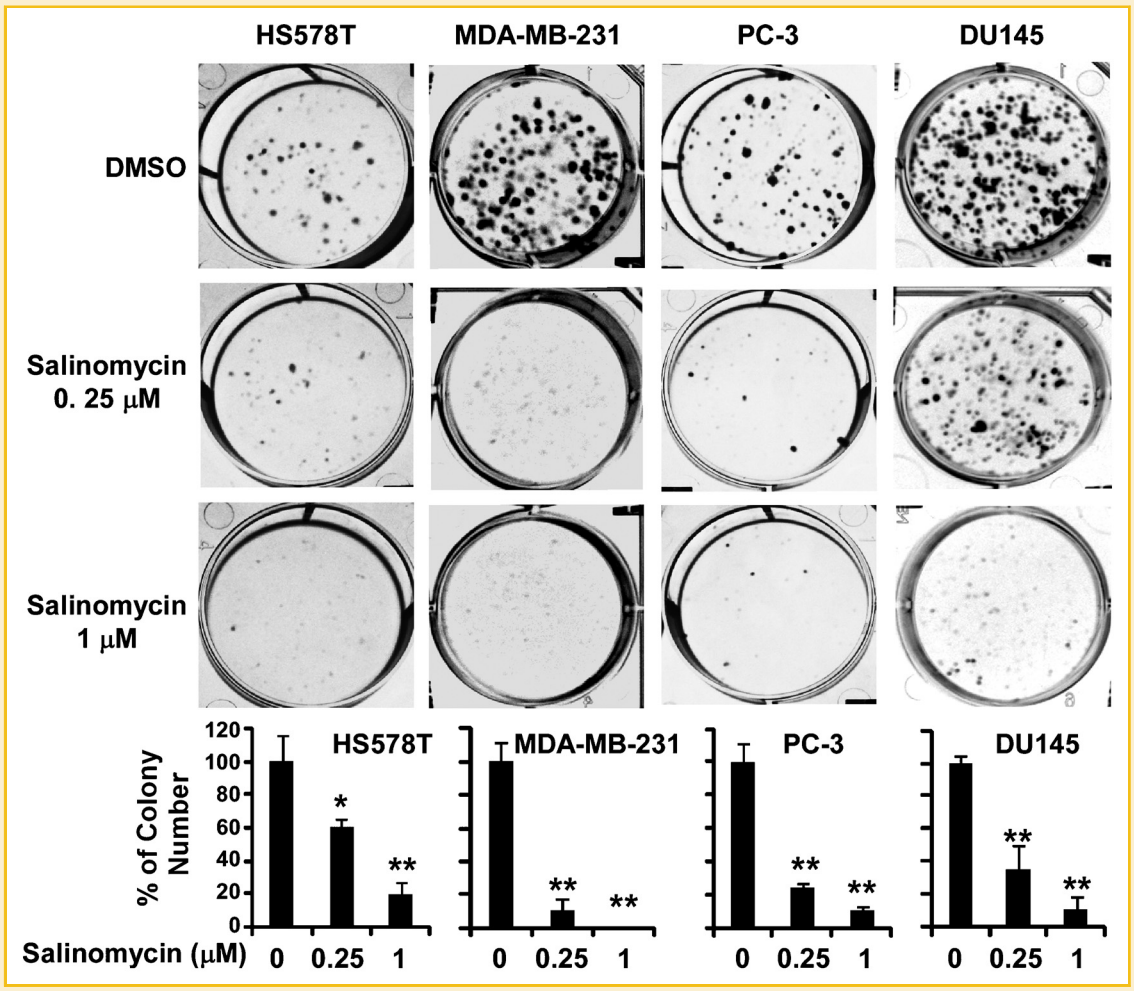

Fig. 8. Salinomycin inhibits cancer cell colony formation. Breast cancer HS578T and MDA-MB-231 cells and prostate cancer PC-3 and DU145 cells in six-well plates were treated with salinomycin for 10–14 days. The media were changed every three days. Colonies were fixed with formaldehyde and stained with crystal violet. All the values are the average of triplicate determinations with the s.d. indicated by error bars. *$P < 0.05$, **$P < 0.01$ compared to cells treated with DMSO.

comparable to those required for inhibiting cancer cell proliferation. Our results indicate that the anticancer activity of salinomycin is associated with its inhibitory effects on LRP6 expression and Wnt/β-catenin signaling.

Inhibition of Wnt/β-catenin signaling at the cell surface can result in activation of GSK3β and downregulation of mTORC1 signaling both in vitro and in vivo [Inoki et al., 2006; Huang et al., 2009, 2012; Liu et al., 2012a; Tahir et al., 2013]. LRP6 antagonist Dkk1 inhibited Wnt3A-induced mTORC1 signaling in MC3T3-E1 osteoblast-like cells and C2C12 myoblasts [Inoki et al., 2006]. Moreover, $LRP6^{+/-}$ mice displayed a diminished Wnt-dependent mTORC1 activity in adipose tissues [Liu et al., 2012a]. Importantly, LRP6 depletion resulted in inhibition of mTORC1 signaling in prostate cancer PC-3 cells [Tahir et al., 2013]. In the present study, we demonstrated that LRP6 overexpression induced mTORC1 signaling in breast cancer MCF-7 cells. We further found that salinomycin activated GSK3β and inhibited mTORC1 signaling in prostate and breast cancer cells. Our results suggest that the inhibitory effect of salinomycin on mTORC1 signaling in cancer cells is associated with its effect on LRP6 expression. However, more studies are required to dissect molecule mechanism underlying salinomycin-mediated inhibition of mTORC1 signaling in details in the future.

The expression of LRP6 is tightly regulated on the cell surface by several proteins. The cell-surface transmembrane E3 ubiquitin ligase zinc and ring finger 3 could inhibit Wnt/β-catenin signaling by promoting the turnover of Wnt receptors Fzd and LRP6 [Hao et al., 2012; Koo et al., 2012], while Rap2, a member of the Ras family of small GTP-binding proteins, is essential for the stabilization of LRP6 [Park et al., 2013]. Furthermore, the transmembrane proteins Kremen1 and Kremen2 can form a ternary complex with LRP6 and Dkk1 to induce rapid endocytosis and removal of LRP6 from the cell surface [Mao et al., 2002]. Salinomycin is a potent LRP6 inhibitor by promoting LRP6 degradation [Lu et al., 2011a]. However, the exact mechanism underlying salinomycin-mediated LRP6 turnover remains to be elucidated in the future studies.

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