

## Co-treatment of Salinomycin Sensitizes AZD5363-treated Cancer Cells Through Increased Apoptosis

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**Abstract.** AZD5363, an inhibitor of protein kinase B (AKT), is currently in clinical trials assessing the potential of the phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway. The purpose of the present study was to identify conditions that increase the sensitivity of cancer cells to AZD5363. Microscopic examination revealed that treatment of cancer cells with a low concentration of salinomycin reduced cellular growth of AZD5363-treated breast cancer cells. Furthermore, fluorescence-activated cell sorting (FACS) analysis, Hoechst staining, and annexin V staining revealed that co-treatment with salinomycin sensitizes AZD5363-treated cancer cells via increased apoptosis with S-phase arrest. These results suggest that salinomycin can be applied to increase treatment efficacy for AZD5363-treated cancer cells. Our findings may contribute to improving the efficacy of the development of AZD5363-based sensitization therapies for patients with cancer.

AZD5363 is an oral small-molecule new-generation drug potentially inhibiting all three isoforms of the protein kinase B (AKT) proteins AKT1, AKT2, and AKT3 (1-4). As an anticancer agent, AZD5363 is being tested in various tumor models such as breast cancer, prostate cancer, and glioma (5-17). In addition, recent clinical trials are investigating the use of AZD5363 in patients with advanced solid tumors. Various synergistic studies were also tested to increase AZD5363 efficacy (5, 7-9, 12-17). For example, cancer cell sensitization to AZD5363 increases after combination with inhibitors of autophagy, insulin-like growth factor I/insulin receptor, phosphoinositide 3-kinase (PI3K), anti-androgen drugs, and drugs such as trastuzumab, enzalutamide, and gefitinib. A thorough understanding of the conditions and mechanisms governing

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AZD5363 sensitization is required to facilitate its therapeutic use in patients with cancer.

Salinomycin was originally used to eliminate bacteria, fungi, and parasites (18). More recently, this drug has been exploited to inhibit the growth of tumor stem cells and is considered a potential anticancer drug for cancer chemoprevention (19-21). Various salinomycin sensitization mechanisms for cancer have also been investigated (19-22). Salinomycin sensitizes cancer cells to doxorubicin, etoposide, radiation, anti-mitotic drugs, and targeting drugs (23-25). Various synergistic studies on salinomycin sensitization mechanisms for cancer have also been investigated (23-28).

In the present study, we aimed to identify conditions that increase AZD5363 efficiency.

### Materials and Methods

*Reagents.* Salinomycin was purchased from Sigma-Aldrich (St. Louis, MO, USA). AZD5363 was supplied by Selleckchem (Houston, TX, USA). LY294002, a potent inhibitor of phosphoinositide 3-kinases (PI3Ks), was supplied by Calbiochem (Bellerica, MA, USA).

*Antibodies.* Antibodies against phosphorylated tuberous sclerosis complex 2 (TSC2), phosphorylated ribosomal protein S6 kinase beta-1 (p70S6K), phosphorylated eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1), proliferating cell nuclear antigen (PCNA), and cleaved poly ADP ribose polymerase (C-PARP) were from Cell Signaling Technology (Danvers, MA, USA). Antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH), survivin, and phosphorylated retinoblastoma protein (Rb) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against phosphorylated mammalian target of rapamycin (mTOR) was from Abcam (Cambridge, UK). Antibody against cyclin D1 was from Biosource (Camarillo, CA, USA).

*Cell culture.* Hs578T breast cancer cells were obtained from the Korean Cell Line Bank (Seoul, South Korea), and were used previously (23-28). The cells were cultured in RPMI-1640 containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (WelGENE, Daegu, South Korea). Hs578T cells grown on 6-well plates or 60 mm-diameter dishes and treated with salinomycin, AZD5363, AZD5363 with salinomycin, or dimethylsulfoxide (DMSO) for 24h or 48h.

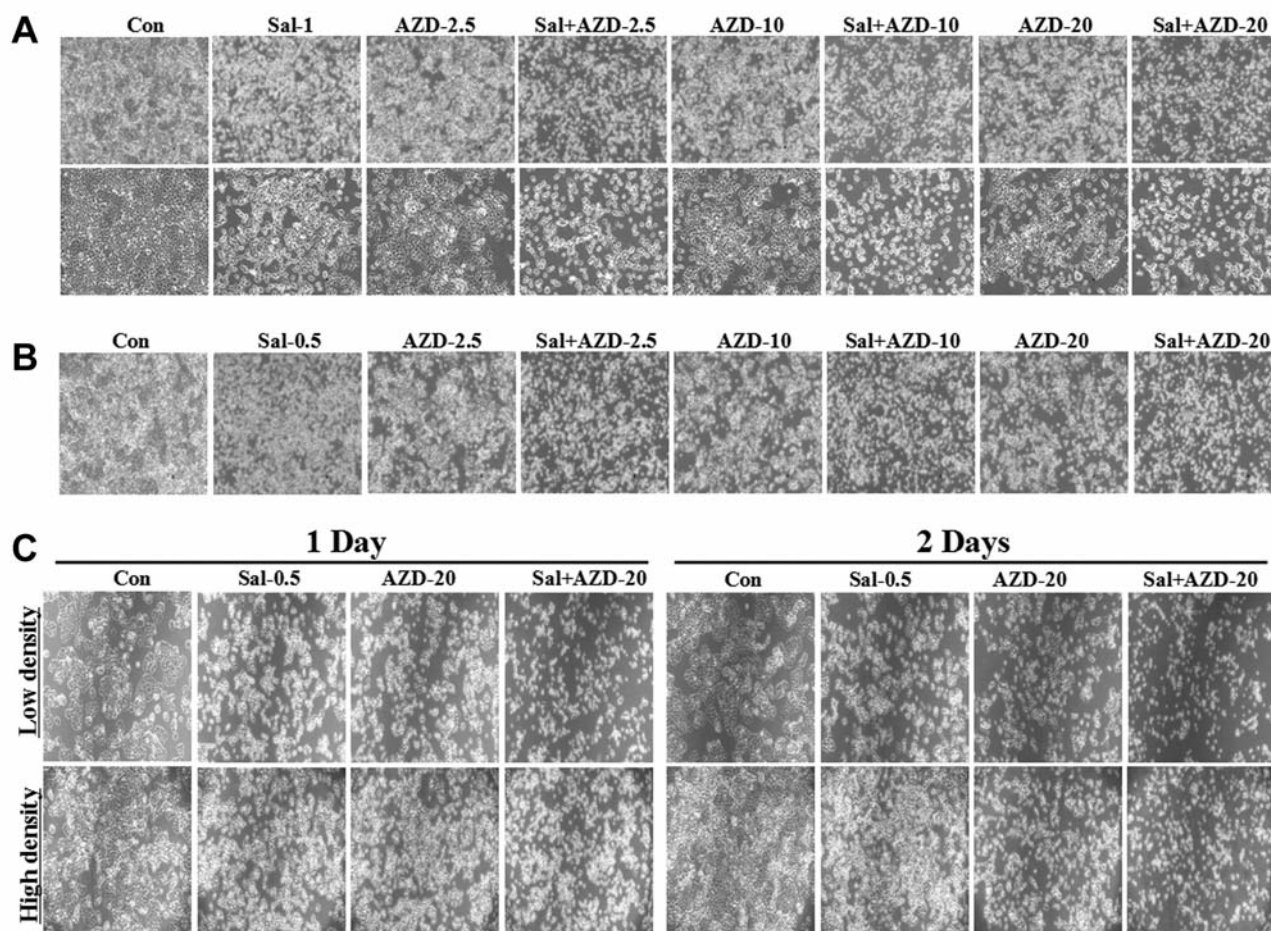


Figure 1. Co-treatment with salinomycin and AZD5363 showing reduced proliferation under different conditions. A: Hs578T breast cancer cells were plated on 6-well plates and treated with 1  $\mu$ M salinomycin (Sal-1), 2.5  $\mu$ M AZD5363 (AZD-2.5), 10  $\mu$ M AZD5363 (AZD-10), 20  $\mu$ M AZD5363 (AZD-20), 2.5  $\mu$ M AZD5363 with 1  $\mu$ M salinomycin (Sal+AZD-2.5), 10  $\mu$ M AZD5363 with 1  $\mu$ M salinomycin (Sal+AZD-10), 20  $\mu$ M AZD5363 with 1  $\mu$ M salinomycin (Sal+AZD-20), or 0.1% dimethylsulfoxide (Con). After two days, all cells were observed using an inverted microscope with  $\times 50$  or  $\times 100$  magnification. We performed two independent experiments. B: Hs578T cells were grown on 6-well plates and treated with 0.5  $\mu$ M salinomycin (Sal-0.5), 2.5  $\mu$ M AZD5363 (AZD-2.5), 10  $\mu$ M AZD5363 (AZD-10), 20  $\mu$ M AZD5363 (AZD-20), 2.5  $\mu$ M AZD5363 with 0.5  $\mu$ M salinomycin (Sal+AZD-2.5), 10  $\mu$ M AZD5363 with 0.5  $\mu$ M salinomycin (Sal+AZD-10), 20  $\mu$ M AZD5363 with 0.5  $\mu$ M salinomycin (Sal+AZD-20), or 0.1% dimethylsulfoxide (Con). After 2 days, all cells were observed using an inverted microscope with  $\times 50$  magnification. We performed two independent experiments. C: Hs578T cells were plated on 6-well plates at a low density or high density with  $1 \times 10^5$ – $3 \times 10^5$  cells/well. The cells were then treated with 0.5  $\mu$ M salinomycin (Sal-0.5), 20  $\mu$ M AZD5363 (AZD-20), 20  $\mu$ M AZD5363 with 0.5  $\mu$ M salinomycin (Sal+AZD-20), or 0.1% dimethylsulfoxide (Con). After one or two days, all cells were observed using an inverted microscope with  $\times 50$  magnification. We performed two independent experiments.

**Western blot analysis.** Total cellular proteins were extracted using a previously described trichloroacetic acid (TCA) method (23-28). Briefly, cells grown in 60-mm dishes were washed three times with 5 ml phosphate buffered saline (PBS). Next, 500  $\mu$ l of 20% TCA were added to each plate. The cells were then dislodged by scraping and transferred to Eppendorf tubes. Proteins were pelleted by centrifugation for 5 min at 600  $\times$ g and resuspended in 1 M Tris-HCl (pH 8.0) buffer. The total protein concentrations were estimated. The proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to western blot analysis as previously described (23-28).

**Fluorescence-activated cell sorting (FACS) analysis.** FACS analysis was performed as previously described (23-28). Cells were grown in 60-mm dishes and treated with salinomycin, AZD5363, AZD5363 with salinomycin, or dimethylsulfoxide (DMSO) for 48h. The cells were then dislodged by trypsin and pelleted by centrifugation. The pelleted cells were washed thoroughly with PBS, suspended in 75% ethanol for at least 1 h at 4°C, washed again with PBS, and re-suspended in a cold propidium iodide (PI) staining solution (100  $\mu$ g/ml RNase A and 50  $\mu$ g/ml PI in PBS) for 30 min at 37°C. The stained cells were analyzed for relative DNA content using a FACSCalibur flow cytometric system (BD

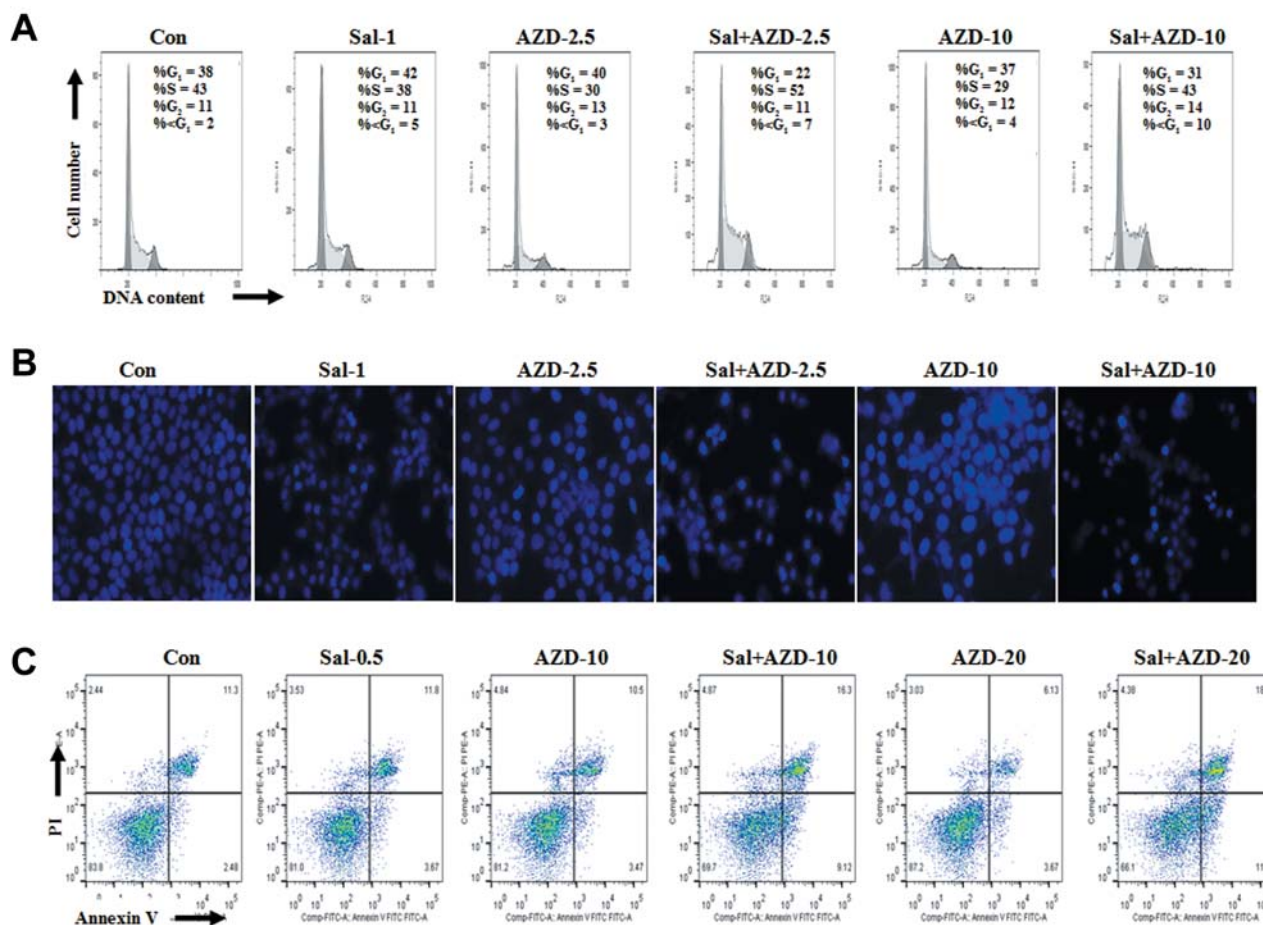


Figure 2. Co-treatment with salinomycin and AZD5363 showing increased apoptosis. **A:** Hs578T cells grown on 60-mm diameter dishes and treated with 1  $\mu$ M salinomycin (Sal-1), 2.5  $\mu$ M AZD5363 (AZD-2.5), 10  $\mu$ M AZD5363 (AZD-10), 2.5  $\mu$ M AZD5363 with 1  $\mu$ M salinomycin (Sal+AZD-2.5), 10  $\mu$ M AZD5363 with 1  $\mu$ M salinomycin (Sal+AZD-10), or 0.1% dimethylsulfoxide (Con). After 48 h, fluorescence-activated cell sorting (FACS) analysis was performed as described in the Materials and Methods. **B:** Hs578T cells were plated on 60-mm diameter dishes. The cells were then grown on 6-well plates and treated as described above. After 48 h, all cells were then stained with Hoechst as described in the Materials and Methods. The stained cells were subsequently examined using an inverted fluorescence microscope with 320 magnification. **C:** Hs578T cells were grown on 6-well plates and treated with 0.5  $\mu$ M salinomycin (Sal-0.5), 10  $\mu$ M AZD5363 (AZD-10), 20  $\mu$ M AZD5363 (AZD-20), 10  $\mu$ M AZD5363 with 0.5  $\mu$ M salinomycin (Sal+AZD-10), 20  $\mu$ M AZD5363 with 0.5  $\mu$ M salinomycin (Sal+AZD-20), or 0.1% dimethylsulfoxide (Con). After 48 h, annexin V analysis was performed as described in the Materials and Methods.

Bioscience, Franklin Lakes, NJ, USA). We performed more than two independent tests.

**Hoechst staining.** These tests were used to identify nuclear disruption, an indicator of apoptosis. Briefly, cells in 6-well plates were treated with drugs as described above and incubated for 48 h at 37°C. Cells were then incubated with 9.4  $\mu$ M Hoechst 33258 (Sigma-Aldrich) for 30 min in the dark at 37°C before image acquisition. The medium was removed, and cells were washed twice with PBS. Stained cells were subsequently examined using an inverted fluorescence microscope. Cellular apoptosis was measured with marked morphological changes such as

condensation of chromatin and nuclear fragmentation. We performed more than two independent tests.

**Annexin V analysis.** Annexin V analysis was conducted using annexin V-fluorescein isothiocyanate (FITC) staining kit (BD Bioscience), as previously described (23-28). Cells were grown in 60-mm diameter dishes and treated with drugs as described above. The cells were then dislodged by trypsin and pelleted by centrifugation. The pelleted cells were washed with PBS. Cells in 100  $\mu$ l of binding buffer received 5  $\mu$ l of annexin V-FITC and 5  $\mu$ l of PI, and were then incubated for 15 min at room temperature. The stained cells were analyzed using a FACSCalibur flow

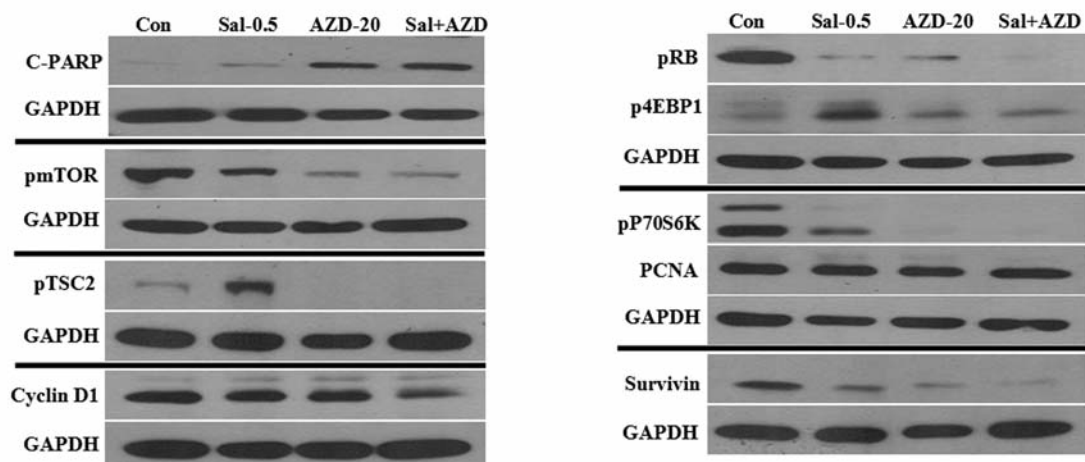


Figure 3. Co-treatment with salinomycin and AZD5363 showing reduced cellular growth signals. Hs578T cell extracts were collected at 24 h after treatment with 0.5  $\mu\text{M}$  salinomycin (Sal-0.5), 20  $\mu\text{M}$  AZD5363 (AZD-20), 20  $\mu\text{M}$  AZD5363 with 0.5  $\mu\text{M}$  salinomycin (Sal+AZD), or 0.1% dimethylsulfoxide (Con). They were then used for western blot analyses using antibodies against cleaved poly ADP ribose polymerase (C-PARP), phosphorylated mammalian target of rapamycin (mTOR), phosphorylated tuberous sclerosis complex 2 (TSC2), cyclin D1, phosphorylated retinoblastoma protein (Rb), phosphorylated eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1), phosphorylated ribosomal protein S6 kinase beta-1 (p70S6K), proliferating cell nuclear antigen (PCNA), survivin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

cytometric system (BD Bioscience). We performed two independent experiments.

## Results

*Co-treatment with salinomycin sensitizes AZD5363-treated breast cancer cells.* The potential of salinomycin to sensitize AZD5363-treated Hs578T breast cancer cells was investigated. Using a relatively low amount of salinomycin, we tested whether AZD5363-treated cells can be further sensitized by salinomycin co-treatment. Microscopic examinations revealed that salinomycin increased sensitization of cells co-treated with different amounts of AZD5363 compared to individual treatment with salinomycin or AZD5363 (Figure 1A). Both 2.5  $\mu\text{M}$  and 10  $\mu\text{M}$  AZD5363 co-treatments with salinomycin showed similar levels of sensitization to 20  $\mu\text{M}$  AZD5363 co-treatment, whereas single-treatment of AZD5363 showed dose-dependent increase in sensitizations. This suggests that a low amount of AZD5363 is enough to sensitize cancer cells when combined with salinomycin. Although 1  $\mu\text{M}$  salinomycin is a relatively low concentration, we employed a concentration as low as 0.5  $\mu\text{M}$  for co-treatment with AZD5363. We found that the 0.5  $\mu\text{M}$  salinomycin also increased sensitization similar to 1  $\mu\text{M}$  salinomycin (Figure 1B). This suggests that salinomycin sensitization with AZD5363 can be achieved with a relatively low risk of toxicity.

The sensitization owing to single treatments of salinomycin and AZD5363 is dependent on cellular density, wherein sensitization responses to the drugs decreased with higher-density cell culture (Figure 1C). It is possible that co-treatment can also be affected by cellular density. However, we observed that co-treatments with salinomycin and AZD5363 were not affected by cellular density within one and two days of treatment (Figure 1C). Collectively, our results indicate that salinomycin treatment can increase the sensitivity of cancer cells to AZD5363 independent of cellular density.

*Co-treatment with salinomycin and AZD5363 increases apoptosis via S-phase arrest.* We further investigated whether reduced proliferation induced by salinomycin and AZD5363 co-treatment involves apoptosis. Co-treatment with salinomycin and AZD5363 increased pre-G<sub>1</sub> cell phases in a dose-dependent manner (Figure 2A), suggesting that co-treatment with salinomycin led to an increase in the apoptosis of AZD5363-treated cells. Further analysis of individual and co-treatment data revealed that co-treatment increased S-phase arrest of cells (Figure 2A), suggesting that sensitization increases apoptosis *via* this mechanism. Hoechst staining was performed to confirm increased apoptosis after co-treatment; staining revealed marked morphological changes characteristic of apoptosis, such as condensation of chromatin and nuclear fragmentation in co-treated cancer cells (Figure 2B).

Consistent with Figure 1A, reduced cell numbers were also observed after co-treatment with salinomycin and AZD5363 (Figure 2B). We also tested whether co-treatment increased staining of annexin V in outer membranes, which is representative of increased apoptosis. The results showed that annexin V staining increased after the co-treatment with salinomycin and AZD5363 (Figure 2C). An in-depth analysis revealed that the population of annexin V-positive only cells in the co-treatments was higher than annexin V- and PI-positive cells (Figure 2C). Considering that cells positive for annexin V only are representative of early apoptosis and annexin V- and PI-stained cells indicate late apoptosis, we conclude that the co-treatments induced more pronounced increases in early apoptosis. In addition, Figure 3, a late apoptotic marker, C-PARP, did not increase after co-treatment with salinomycin and AZD5363. These data confirm that co-treatment with salinomycin increases early apoptosis of AZD5363-treated cancer cells.

We further analyzed whether the co-treatment influenced the activation status or levels of the signaling proteins that function upstream and downstream of the PI3K/AKT/mTOR pathway (26, 27, 29, 30). We analyzed phosphorylated forms of the major proteins, mTOR, P70S6K, 4EBP1, and TSC2. Figure 2B, the activation states of all the proteins were similar after single AZD5363 treatment and co-treatment. Since the PI3K/AKT/mTOR pathway is involved in proliferation and survival signals (26, 27, 29, 30), we also tested whether the levels of cell cycle- and proliferation-related proteins (cyclin D1, pRb, PCNA, and survivin) were altered. As seen in Figure 3, all protein levels were similar after single treatment with AZD5363 and co-treatment. These results suggest that single treatment with AZD5363 is enough to reduce the levels of these growth signaling pathway proteins. In addition, co-treatment with salinomycin and AZD5363 abrogated the increase of pTSC2 and p4EBP1 levels induced by single salinomycin treatment (Figure 3). This suggests that co-treatment of AZD5363 with salinomycin can increase sensitization through attenuation of growth signals.

Collectively, our results indicate that increased apoptosis is observed when salinomycin is combined with AZD5363 *via* increased S-phase arrest and reduced AKT pathway signaling.

## Discussion

AZD5363 is a recently developed drug that inhibits AKT isoforms (1-4). In the present study, we attempted to identify ways to enhance sensitization of AZD5363-treated cancer cells. We showed that AZD5363 increases sensitization when used in combination with salinomycin. We also demonstrated that 2.5  $\mu$ M AZD5363 was enough to induce a level of

growth inhibition similar to 20  $\mu$ M AZD5363 when used with salinomycin, suggesting that co-treatment with salinomycin can reduce AZD5363 toxicity with its relatively low dose. Since a relatively low amount of salinomycin was used in this study, salinomycin toxicity can be reduced by combining treatment with AZD5363 in future clinical trials.

Sensitization of cancer cells to various anticancer drugs and radiation by co-treatments with salinomycin has been demonstrated (19-28). However, the sensitization of cancer cells to specific molecular-targeting drugs using salinomycin has not yet been determined. The sensitization of MK-2206-treated cells by salinomycin co-treatment has been reported (26). Since we also reported that AZD5363 could increase sensitization of salinomycin-treated cells, we suggest that salinomycin may also be useful in combination with various specific AKT molecule-targeting drugs. The fact that sensitization is observed when combining salinomycin with either AZD5363 or MK-2206 suggests that this effect may be conserved among AKT inhibitors, possibly proposing that various AKT inhibitors could be combined with salinomycin for sensitization.

Our data indicate that the mechanism sensitization involves apoptosis since an increase in the proportion of pre-G<sub>1</sub> region cells was detected and confirmed by both Hoechst and annexin V staining. In particular, early apoptotic events were more pronounced than late apoptotic events. We also determined that S-phase arrest was involved in the sensitization induced by co-treatments. We earlier hypothesized that AZD5363-treated cancer cells can be sensitized with salinomycin by affecting important AKT or proliferation signaling molecules; however, the levels of important proteins were similar in cells with single AZD5363 treatment and co-treatment. These results suggest that single treatment with AZD5363 is enough to reduce growth signaling pathway proteins. However, we did not identify specific molecules reduced more by co-treatments. This suggests that further analysis is needed for molecular targeting for other possible co-treatments.

In summary, our results could help in determining the potential clinical use of salinomycin for AZD5363-treated patients with cancer. The present study also enhances our understanding over salinomycin-sensitization mechanisms. Our findings may contribute to the development of AZD5363-based therapies for patients.

## Conflicts of Interest

The Authors declare no conflicts of interest.

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