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Generation and Characteristics of A Salinomycin-Resistant Breast Cancer Cell Line

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Abstract

Understanding salinomycin (Sal)-resistance is essential for its further clinical use in patients. Thus, we generated a relatively Sal-resistant cancer cell line (SAL-Res) from Sal-sensitive Hs578T breast cancer cells. Characteristics of SAL-Res cells were then analyzed in comparison to those of Hs578T parent cells. Although we observed that SAL-Res and Hs578T cells presented a similar growth phenotype, we found that SAL-Res cells easily detached by shaking or trypsinization when compared to Hs578T cells. It suggests that the attachment ability of SAL-Res cells on the surface is different from those of Hs578T cells.

Furthermore, we tried to identify sensitizing conditions that increase the sensitivity of SAL-Res cancer cells to various clinical chemotherapeutic drugs. The results showed that SAL-Res and Hs578T cells present similar sensitivity to chemotherapeutic drugs, suggesting that SAL-Res cells do not acquire their resistant phenotype through other anti-cancer drugs. It also indicates that SAL-Res can be easily killed by various anti-cancer drugs, suggesting that Sal may be the preferred drug to be used for the treatment of patients with cancer. We also demonstrated that both SAL-Res and Hs578T cells present similar drug-efflux ability, suggesting that SAL-Res cell resistance mechanism does not depend on the inhibition of p-glycoprotein (P-gp) and may be related to cellular signaling pathways. Our findings may contribute to the development of Sal-based sensitization therapies for patients with cancer.

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Introduction

Salinomycin (Sal) was originally used to eliminate bacteria, fungi, and parasites [1,2]. More recently, this drug has been exploited to inhibit the growth of tumor stem cells and chemoresistant cancer cells [3,12]. Sal also functions as an efflux pump p-glycoprotein (P-gp) inhibitor [4,13,14] and is considered to be a potential anti-cancer drug for cancer chemoprevention. Additionally, Sal sensitizes cancer cells to doxorubicin, etoposide, radiation, and anti-mitotic drugs [14-16]. Various Sal-sensitization mechanisms for cancer have also been investigated [17-25]. It has been investigated as an anti-cancer drug in clinical trials assessing the potential of pAkt targeting therapy [26,27]. A more complete understanding of the mechanisms governing Sal sensitization is required to facilitate its therapeutic use to treat patients with cancer. Since it is assumed that patients develop resistance to Sal, it would be important to determine Sal-resistant cancer cell characteristics before the establishment of Sal as a routine clinical treatment for patients with cancer. Identifying the cellular mechanism(s) underlying the resistance to Sal would be an important step in the development of new treatment methods for patients with cancer.

In the present study, we generated a Sal-resistant cancer cell line (SAL-Res) and confirmed that SAL-Res cells are relatively resistant to Sal treatment. We also identified specific features of SAL-Res cells, which are different from those of Hs578T breast cancer parent cells. We also identify anti-cancer drugs that can sensitize SAL-Res cells. Our results may contribute to the development of a Sal-based therapy for patients with cancer.

Materials and Methods

Reagents Aqueous solutions of doxorubicin (Boryung Pharmacy, Seoul, South Korea), was obtained from the National Cancer Center in South Korea. Sal, etoposide, and verapamil were purchased from Sigma-Aldrich (St. Louis, MO). Hydroxyurea and Rhodamine123 (Rhodamine) was purchased from Santa Cruz Biotechnology (Dallas, Texas). Vincristine, vinorelbine, and daunorubicin were purchased from Enzo Life Sciences (Farmingdale, NY, USA). Calcein-AM was obtained from Invitrogen (Carlsbad, CA, USA).

Cell culture

Hs578T breast cancer cells were obtained from the Korean Cell Line Bank (Seoul, South Korea) and were previously used [16, 26, 28]. SAL-Res cells were generated from Hs578T cells, which were continuously grown in the presence of 0.1 μM Sal, as a starting concentration. Cells were trypsinized and transferred to new dishes upon reaching 70-80% confluence. Sal concentration was increased approximately every 2-3 weeks, once the cells were growing at a similar rate. The concentration was increased up to 3 μM. After 6 months of culture, the cells finally presented a resistant phenotype. Sal-resistant cells were defined as those growing well after a two day treatment with 1 μM Sal, whereas sensitive Hs578T parental cells were dying under the same conditions.

Human oral squamous carcinoma cell line, KB, and its multidrug-resistant subline, KBV20C, were obtained from Dr. Yong Kee Kim and were previously described [29]. All cell lines were cultured in RPMI 1640 or DMEM containing 10% fetal bovine serum, 100U/mL penicillin, and 100 μg/mL streptomycin (WelGENE, Daegu, South Korea).

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Rhodamine and Calcein-AM uptake tests

These tests were used to determine the ability to inhibit P-gp with the indicated drugs by using a previously described method [30-32]. Briefly, cells grown in 6-well plates were treated with the indicated drugs and incubated for 24 h at 37°C. Cells were then incubated with 1 μg/mL Rhodamine or 0.1 μg/mL Calcein-AM for 1 h 30 min at 37°C. The medium was removed, and the cells were washed with PBS. Stained cells were analyzed using a FACSCalibur flow cytometry system (BD Biosciences, Franklin Lakes, NJ). In this experiment, cellular accumulation of green fluorescence was indicative of Calcein-AM and Rhodamine intracellular accumulation. We performed two independent experiments.

Microscopic observation

Cells grown in 6-well plates were treated with the indicated drugs for the indicated times. The medium was removed, and PBS was added in each dish. Cells were examined immediately using an Axio observer.Z1 fluorescence inverted microscope (Carl Zeiss, Oberkochen, Germany) with a 5× or 10× objective lens (Carl Zeiss EC Plan-Neofluar). We performed two independent experiments.

Results

Generation of the Sal-resistant breast cancer cell line

Continuous treatment of cancer patients with Sal induces the development of Sal-resistant cancer cells through anti-cancer drug-resistance mechanisms (30-34). Thus, understanding Sal-resistant cancer cell characteristics is essential before using it for routine cancer treatment in the clinic. We generated a relatively Sal-resistant cancer cell line, SAL-Res. SAL-Res cancer cell line was derived from the Hs578T breast cancer cell line, which was cultured with increasing concentrations of Sal over a period of 6 months (Figure 1A). Characteristics of SAL-Res cells were then analyzed and compared to those of Hs578T cells. As shown in Figure 1A, SAL-Res cells developed a resistant phenotype when treated with 1 μM Sal for 1 and 2 days. The sensitization difference between SAL-Res and Hs578T cells were more obvious after 2 days of Sal treatment than after 1 day of treatment, suggesting that the Sal effect is long-lasting only in sensitive Hs578T cells. Sal has previously been shown to sensitize cells with a similar degree in both high- and low-density cellular populations [18]. However, at a higher density, SAL-Res cells presented a resistant phenotype after Sal treatment, whereas Hs578T cells were sensitized similarly at both high and low densities (Figure 1A). These results suggest that SAL-Res cells present a different phenotype than their Hs578T parent cells.

Although SAL-Res cell line was generated by culturing the cells in up to 3 μM Sal, SAL-Res cells could still be sensitized in the presence of 2 or 3 μM Sal treatment when cultured at a high density (Figure 1B). When SAL-Res cell line was generated after 6 months of culture, it was hard to increase the concentration of Sal above 3 μM. These data suggest that it is difficult to acquire Sal-resistance. We assumed that the Sal-resistance mechanism might not exist, or that Sal-resistance can be easily reversed by modifying the culture conditions, or that Sal-resistance is not long lasting.

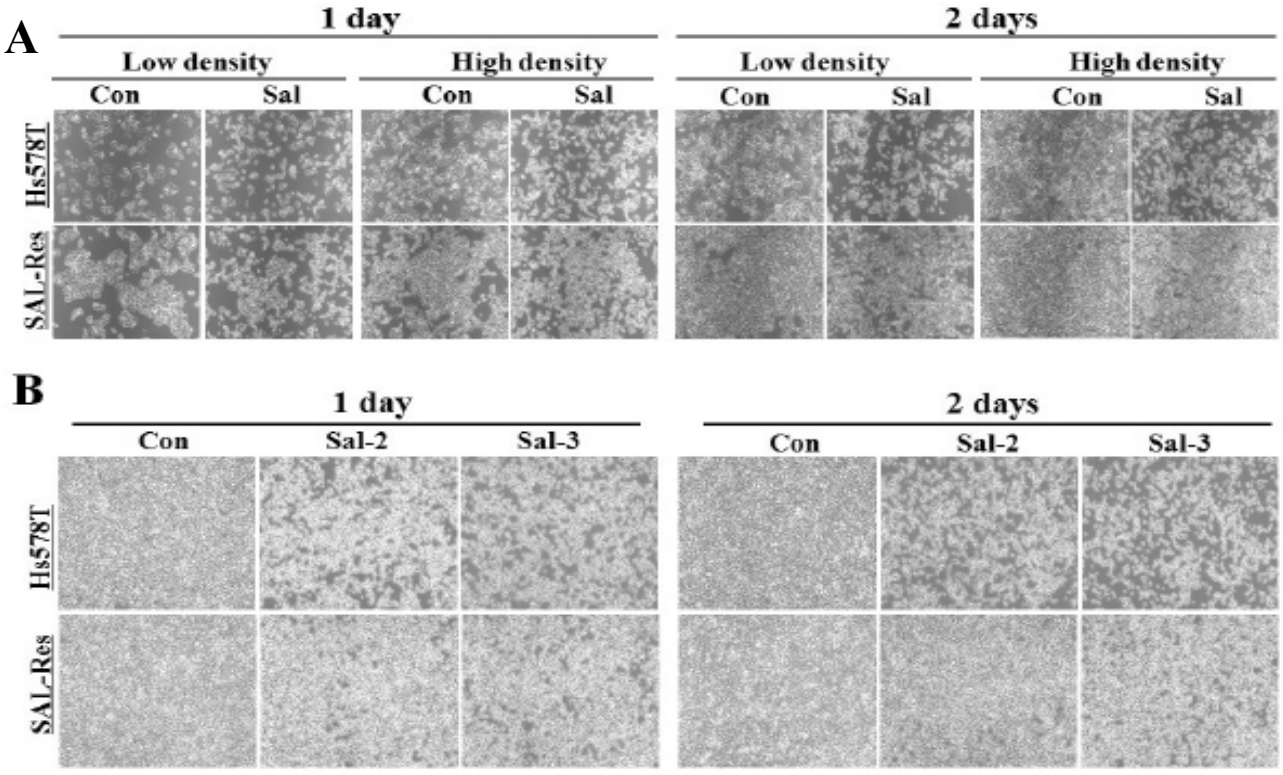

Figure 1: Compared to Hs578T parent cells, SAL-Res cells are relatively resistant to Sal treatment
(A) Hs578T and SAL-Res cells with 1.5×10^5 (Low density) or 2.5×10^5 (High density) were plated on 6-well plates. The cells were then grown on 6-well plates and treated with 1 μM Sal (Sal), or 0.1% DMSO (Con). After 1 or 2 days, all cells were observed using an inverted microscope with a 5X objective lens. We performed two independent experiments. (B) Hs578T and SAL-Res cells were grown on 6-well plates and then incubated for 1 or 2 days with 2 μM Sal (Sal-2), 3 μM Sal (Sal-3), or 0.1% DMSO (Con). All cells were observed using an inverted microscope with a 5X objective lens. We performed two independent experiments.

Sal-resistant breast cancer cells easily detached from the surface

Next, we examined the mechanisms underlying SAL-Res cell growth. First, SAL-Res cell growth was compared with that of parent Hs578T cells at different cellular densities. As shown in Figure 2A and B, SAL-Res cell growth was similar to that of Hs578T cells. Drug-resistant cancer cells often show slow growth phenotypes. However, SAL-Res cells do not present this phenotype. When we carefully analyzed the results, we observed that, at 1 day with relatively lower density, SAL-Res cells showed a slightly higher growth phenotype than Hs578T cells (Figure 2A). Secondly, it was found that SAL-Res cells presented a lesser binding affinity on surfaces than Hs578T cells (Figure 2C). Since SAL-Res cells were easily detached from the surface by shaking, we assume that the detached cells use the anoikis pathway [19]. The decrease in binding ability was also confirmed by the fact that the trypsinization time for cellular detachment was less for SAL-Res cells than for Hs578T cells (Figure 2D). Collectively, we generated a SAL-Res cell line and determined that this cell line presents a different phenotype when compared to parent Hs578T cells, in terms of growth and cellular attachment strength on surfaces.

Sal-resistant breast cancer cells easily detached from the surface

Drug-resistance cancer cells are usually resistant to other anti-cancer drugs [30-32]. Thus, we further analyzed which anti-cancer drugs can sensitize SAL-Res cells. SAL-Res and Hs578T cell sensitivity was compared side by side. Parents Hs578T cells presented a much resistant phenotype. We tested various clinical anti-cancer drugs, which are currently used to treat patients with various cancers. In this analysis, we included DNA-damaging drugs such as doxorubicin, etoposide, and daunorubicin. Anti-mitotic drugs such as vincristine and vinorelbine were also tested included along with hydroxyurea.

As shown in Figure 3A-C, most anti-cancer drugs had similar sensitization effects in both SAL-Res and Hs578T parent cells, whereas SAL-Res cells were resistant to Sal treatment. Although the concentration was increased or reduced, the sensitization-effect difference between SAL-Res and Hs578T cells was similar (Figure 3A-C). In conclusion, when compared to Hs578T parent cells, SAL-Res cells showed similar sensitivity to most anti-cancer drugs, while they acquired resistance to Sal treatment, suggesting that SAL-Res cells present a specific resistance for a drug, Sal.

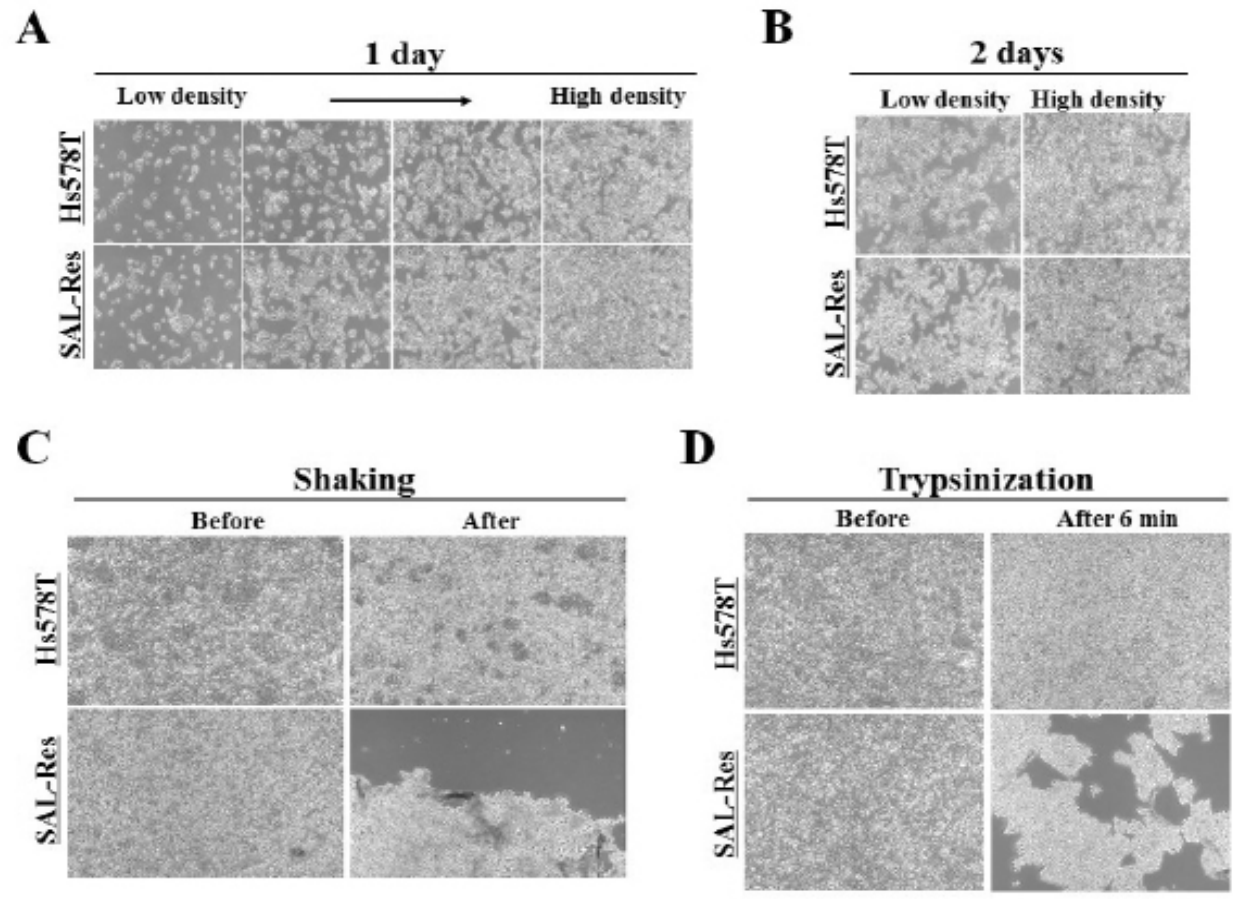

Figure 2: SAL-Res cells grow as fast as Hs578T cells do, but are easily detachable

(A) Hs578T and SAL-Res cells with four different cell densities (Low to High density) were plated on 6-well plates. The cells were then grown on 6-well plates for 1 day. All cells were observed using an inverted microscope with a 5× objective lens. We performed two independent experiments. (B) Hs578T and SAL-Res cells with 0.5 × 105 (Low density) or 1 × 10^5 (High density) were plated on 6-well plates. The cells were then grown on 6-well plates for 2 days. All cells were observed using an inverted microscope with a 5× objective lens. We performed two independent experiments. (C) Hs578T and SAL-Res cells were grown on 6-well plates and then incubated for 3 days. The medium was removed and PBS was added. Cells were then shaken gently. All cells were observed using an inverted microscope with a 5× objective lens. We performed two independent experiments. (D) Hs578T and SAL-Res cells were grown on 6-well plates and then incubated for 2 days. The medium was removed and PBS was added. Cells were then trypsinized. After 6 min at room temperature, all cells were observed using an inverted microscope with a 5× objective lens. We performed two independent experiments.

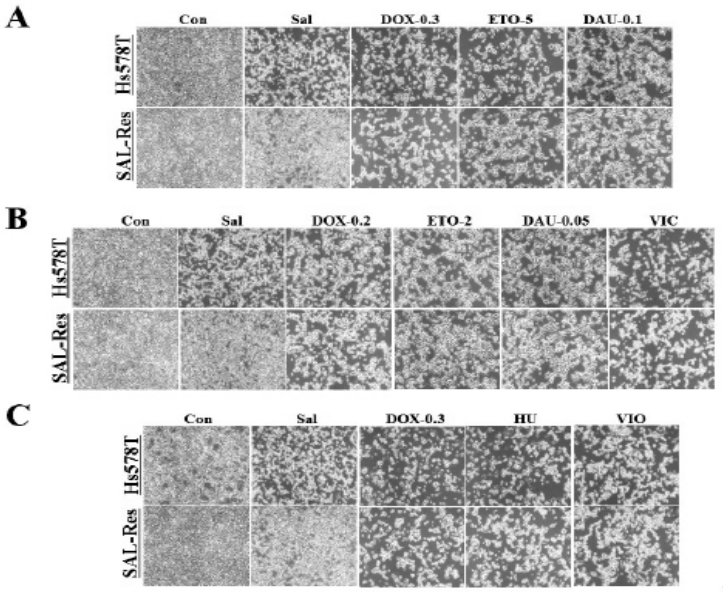

Figure 3: Both Sal-resistant and Hs578T parent cells are sensitized by various anti-cancer drugs

(A-C) Hs578T and SAL-Res cells were grown on 6-well plates and treated with 2 μM Sal (Sal), 0.3 μM doxorubicin (DOX-0.3), 0.2 μM doxorubicin (DOX-0.2), 5 μM etoposide (ETO-5), 2 μM etoposide (ETO-2), 0.1 μM daunorubicin (DAU-0.1), 0.05 μM daunorubicin (DAU-0.05), 10 nM vincristine (VIC), 1 mM hydroxyurea (HU), 0.1 μg/mL vinorelbine (VIO), or 0.1% DMSO (Con). After 2 days, all cells were observed using an inverted microscope with a 5× objective lens. We performed two independent experiments.

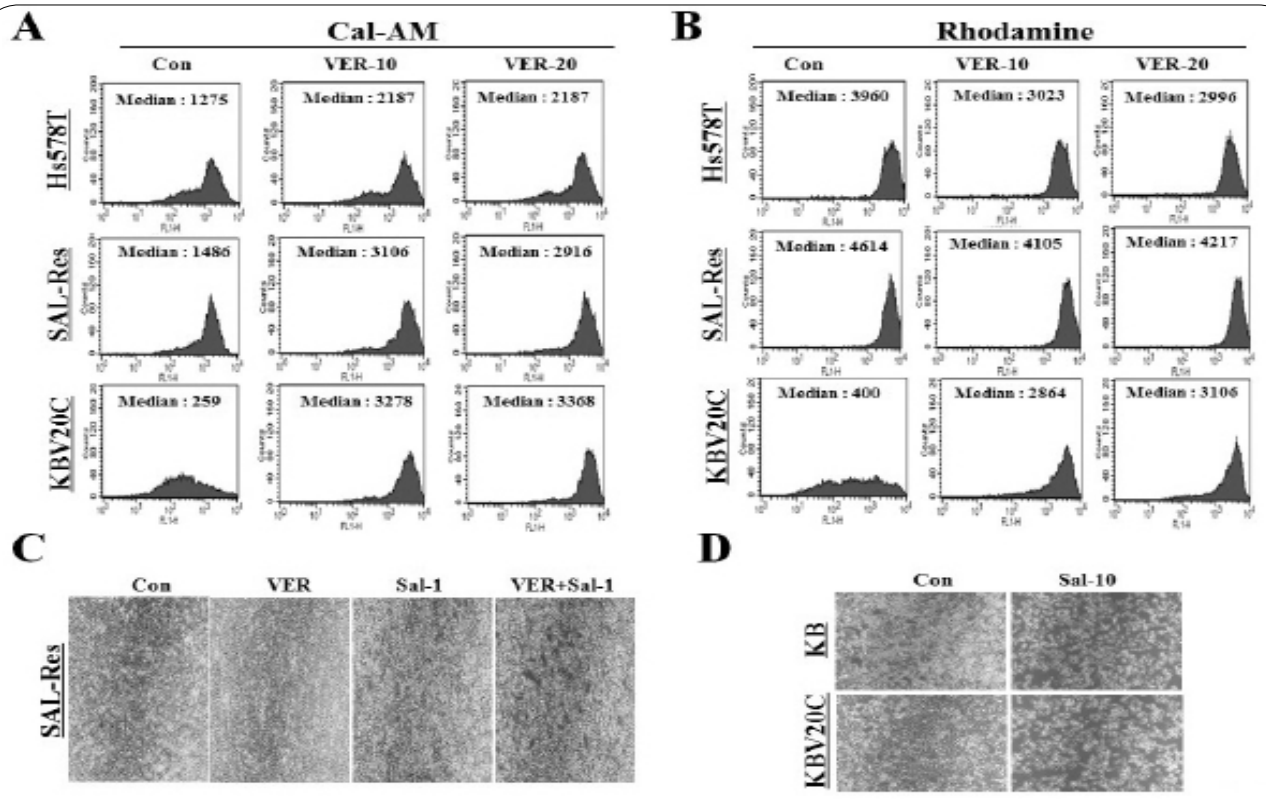

Figure 4: SAL-Res cells do not acquire the pumping-out ability via P-gp

(A-B) Hs578T, SAL-Res, and KBV20C cells were grown on 6-well plates and treated with 10 μM verapamil (VER-10), 20 μM verapamil (VER-20), or 0.1% DMSO (Con). After 24 h, all cells were stained with Cal-AM or Rhodamine, as described in Materials and Methods. The stained cells were subsequently examined by using FACS analysis. (C) SAL-Res cells were grown on 6-well plates and treated with 10 μM verapamil (VER), 1 μM Sal (Sal-1), 10 μM Sal (Sal-10), 10 μM verapamil + 1 μM Sal (VER+Sal-1), 10 μM verapamil + 10 μM Sal (VER+Sal-10), or 0.1% DMSO (Con). After 2 days, all cells were observed using an inverted microscope with a 5× objective lens. We performed two independent experiments. (D) KB and KBV20C cells were grown on 6-well plates and were then incubated for 1 day with 10 μM Sal (Sal-10) or 0.1% DMSO (Con). After 2 days, all cells were observed using an inverted microscope with a 5X objective lens. We performed two independent experiments.

SAL-Res cell line does not acquire the pumping-out ability via P-gp

Next, we examined the mechanisms underlying the SAL-Res cell resistance phenotype. Inhibition of membrane efflux of anti-cancer drugs is an important sensitization mechanism for drug-resistant cancer cells [30, 35]. Therefore, we examined whether SAL-Res cells present a P-gp activity. In order to test whether SAL-Res cell resistance depended on P-gp, we tested whether SAL-Res increases P-gp substrate efflux, when compared to Hs578T cells. KBV20C, a P-gp expressing cell line [29], was also tested as a positive control. Both Calcein-AM and Rhodamine, well-known P-gp substrates, were used to measure the efflux [30-32]. The substrate accumulation was similar for both Hs578T and SAL-Res cells, whereas KBV20C cells showed a largely increased substrate efflux (Figure 4A and Figure 4B). Verapamil, a well-known P-gp inhibitor [30-32], only increased substrate accumulation in KBV20C in a concentration dependent manner (Figure 4A and Figure 4B). Although both Hs578T and SAL-Res cells showed a 2-fold increase in Calcein-AM substrate accumulation after verapamil treatment (Figure 4A), the rhodamine efflux was not inhibited by verapamil (Figure 4B). These data indicate that P-gp activity was not acquired during the generation of SAL-Res cells from Hs578T parent cells.

We also tested whether co-treatment of Sal with verapamil increases SAL-Res cell sensitization. KBV20C, well-known P-gp expressing cell line, could be sensitized by co-treatment with verapamil and the anti-cancer drug [29]. However, as shown in Figure 4C, we found that Sal did not increase the verapamil-induced sensitization in SAL-Res cells, suggesting that Sal is not a P-gp substrate. We also observed that drug-sensitive KB cells and resistant KBV20C cells presented similar Sal-sensitivity (Figure 4D). These results suggest that Sal is not a P-gp substrate and support the notion that the SAL-Res cell line resistant phenotype is not linked to P-gp increases. We conclude that SAL-Res cells acquire its Sal-resistant phenotype without efflux pumping-out abilities.

Discussion

During treatment, cancers finally acquire resistance to anti-cancer drugs [33-35]. We assumed that resistance to Sal could also appear when patients with cancer are administered long-term treatment with Sal. Understanding Sal-resistance is essential for its further clinical use in patients. Thus, we generated a SAL-Res cell line from Hs578T breast cancer cells.

We generated the SAL-Res cell line by culturing Hs578T cells in presence of Sal for 6 months. To create Sal-resistant cells, Sal concentration was increased as the cells grew. However, it was difficult to create Sal-Res cells above 3 μM Sal. Although SAL-Res cells were generated with 3 μM Sal, compared to untreated SAL-Res, these cells still presented some sensitivity to 2μM or 3μM Sal. These results suggest that cancer cells do not acquire Sal resistance easily and remain somewhat sensitive to it. It indicates that Sal could be an effective drug for cancer treatment. Additionally, a higher cellular density was required to grow SAL-Res cells in presence of Sal. Sal treatment sensitizes Hs578T cells to a similar degree in either high- or low-density cellular population. However, SAL-Res cell resistance to Sal depends on cellular density, suggesting that Sal-Res cells might present resistance mechanisms when cultured at a high density. These observations make us define the resistant cells as relatively Sal-resistant cancer cells as these cells are not completely resistant to Sal.

SAL-Res cells have some characteristics, which can be distinguished from those of conventional drug-resistant cells. Usually, drug-resistant cancer cells grow slower. However, the growth of SAL-Res cells was very similar to that of Hs578T parent cells, suggesting that SAL-Res cell growth control is not related to the Sal-resistance mechanism. Drug-resistant cancer cells usually present increased P-gp expression, which can pump-out drugs to reduce inside concentrations of toxic drugs [30-35]. However, SAL-Res presented a P-gp activity similar to that of Hs578T parent cells, suggesting that, in SAL-Res cells, Sal efflux is not related with the Sal-resistance mechanism. Interestingly, SAL-Res cells easily detached from the surface, and the detached cells are assumed to die via the anoikis cellular death pathway [19]. It would be interesting to investigate which inside molecules or membrane proteins are involved in the transformation of Hs578T into SAL-Res cells. We assumed that these characteristic could participate in the evolution of Hs5878T into SAL-Res cells to overcome Sal toxicity. It should also be checked whether Sal-resistant cells derived from other organs or cancer cell lines present phenotypes similar to those of SAL-Res cells.

The most important finding was the identification of anti-cancer drugs that sensitize SAL-Res cells. We tested various clinical anti-cancer drugs, which are currently used to treat patients with various cancers. Based on testing over various anti-cancer drugs, we found that most drugs sensitize both parent Hs578T and SAL-Res cells to a similar degree. Since those anti-cancer drugs are targeting various sensitization mechanisms, including DNA damage and anti-mitotic effects, our results suggest that SAL-Res can be sensitized by any type of anti-cancer drug. It also suggests that SAL-Res cells only acquired resistance to Sal. Thus, other anti-cancer drugs can be used to treat patients if Sal-resistance is observed. We assume that these findings are clinically relevant for the application of combination therapies. The present study enhances our understanding of one of several Sal-sensitization mechanisms. Our results could help to determine the potential clinical treatment of patients with Sal-resistant cancer.

Competing Interests

The authors have declared that no competing interests exist.

Author Contributions

All the authors substantially contributed to the study conception and design as well as the acquisition and interpretation of the data and drafting the manuscript.

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