Doctor of Philosophy

A mechanism study of aprepitant, a neurokinin 1 receptor antagonist, for overcoming trastuzumab resistance in HER2-positive breast cancer

HER2 양성 유방암 대상 뉴로키닌 1 수용체 길항제 아프레피턴트의 트라스투주맙 내성 극복 항암 기전 연구

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A mechanism study of aprepitant, a neurokinin 1 receptor antagonist, for overcoming trastuzumab resistance in HER2-positive breast cancer

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Abstract

Despite the use of anti-HER2 therapies, many patients with HER2-positive breast cancer experience relapse or metastatic disease. Based on the observations of the oncogenic properties of substance P (SP) and the neurokinin 1 receptor (NK1R) in breast cancer tumorigenesis including HER2 activation, we investigated the novel effects of aprepitant, a NK1R antagonist that is generally used as an antiemetic drug, in the context of overcoming drug resistance in HER2-positive breast cancer patients. There was a significant reduction in cell viability and induction of apoptosis, accompanied by caspase activation and suppression of MEK/ERK phosphorylation in both trastuzumab-sensitive and -resistant cells, with the use of aprepitant. With regard to HER2 signaling, aprepitant use resulted in an apparent downregulation of HER3 and a notable reduction of truncated p95HER2. Moreover, aprepitant showed the ability to suppress the breast cancer stem cell (BCSC)-like properties in HER2-positive breast cancer cells, as evidenced by a marked inhibition of ALDH1 activity, reduction of the $CD44^{high}/CD24^{low}$ subpopulation, and impairment of mammosphere formation. Although aprepitant was not able to sensitize trastuzumab-resistant cells to trastuzumab, these anticancer effects were also observed in trastuzumab- and lapatinib-resistant JIMT-1 cells, implying that aprepitant could overcome multidrug-resistance. Therefore, the findings of this study suggested that aprepitant could be an attractive candidate for drug repurposing as an

alternative agent for HER2-positive breast cancer patients who have experienced treatment failure with trastuzumab.

Keywords: Drug repurposing, Aprepitant, Breast cancer, HER2 signaling, Cancer stem cell

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Introduction

The human epidermal growth factor receptor 2 (HER2) is a tyrosine kinase receptor that belongs to the epidermal growth factor receptor (EGFR/ErbB) family. It is overexpressed or amplified in 20–30% of invasive breast cancer cases [1]. HER2 has no ligand and is activated through homo- or heterotypic interactions between its extracellular domains and those of other ErbB family members. Since HER2 dimerization facilitates the activation of its tyrosine kinase domain and various downstream intracellular signaling pathways, it is a well-known prognostic marker for worse outcome in patients with breast cancer [2].

The prognosis of HER2-positive breast cancer has shown a marked improved with development of various anti-HER2 agents, including the famous anti-HER2 antibody, trastuzumab. However, approximately one in four patients with HER2-positive early breast cancer (EBC) have been shown to still relapse, despite 1-year of standard adjuvant anti-HER2 therapy. HER2-positive advanced breast cancer (ABC) remains an incurable disease [3]. Several resistance mechanisms have been identified including the expression of HER2 receptor variants (such as the truncated form [p95HER2]), a constitutive activation of the MET or PI3K/AKT/mTOR pathway, an overexpression of HER2 dimerization partners (such as EGFR/HER1 and HER3), the presence of extrinsic signals within the tumor microenvironment, and the rise of self-renewing breast cancer stem cells (BCSC).

Tachykinins, exemplified by substance P (SP), are small neuropeptides present in the

tumor microenvironment. They are synthesized by macrophages, neuronal, endothelial, and epithelial cells, and show a widespread distribution throughout the body [4]. Binding to the neurokinin 1 receptor (NK1R), SP participates in important physiologic processes, including inflammation, nociception, smooth muscle contractility, epithelial secretion, and proliferation [5]. Besides these physiological functions, recent studies have demonstrated the oncogenic properties of the SP-NK1R system via the activation of various intracellular pathways that involve cell proliferation and survival [6]. Moreover, NK1R signaling can trans-activate the receptors belonging the ErbB family, including EGFR and HER2, enhancing their aggressiveness of breast cancer cells [7, 8]. Therefore, blocking SP-NK1 system may be beneficial in the treatment of HER2-positive breast cancer.

Already in clinical practice, NK1 receptor antagonists, such as aprepitant and fosaprepitant, are widely used. Given the role of SP-NK1R in the central emetic circuitry, these were developed originally and approved for chemotherapy-induced and postoperative- nausea and vomiting (CINV or PONV). In the present study, we sought to characterize the mechanisms of action responsible for aprepitant's novel effects in targeting BCSC and the HER2/Akt signaling pathway in HER2-positive breast cancer cell lines, even in those that showed resistance to trastuzumab.

Materials and Methods

1. Reagent and antibodies

Aprepitant was purchased from the Tokyo Chemical Industry Co. (Tokyo, Japan). Trastuzumab (Herceptin®) was purchased from the Korea University Guro Hospital Pharmacy. Triton X-100, propidium iodide (PI), and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (MO, USA). The phosphatase inhibitor and protease inhibitor cocktail tablets were purchased from Roche Applied Sciences (Penzberg, Germany). The primary antibodies that were used targeted the following proteins: Akt, MEK, phospho-MEK (Ser217/221), total-ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), PARP, cleaved-caspase-3, cleaved-caspase-7, cleaved-caspase-8, EGFR, phospho-EGFR, HER2, phospho-HER2 (Tyr1221/1222), HER2, and phospho-HER3 (Tyr1289) (Cell Signaling Technology, CA, USA); the anti-intracellular domain (ICD) HER2 clone CB11 (Thermo Fisher Scientific Fremont, CA, USA); and GAPDH (Sigma-Aldrich, MO, USA). The secondary antibodies that were used were horseradish peroxidase (HRP)-conjugated anti-rabbit and mouse IgG (Bio-Rad Laboratories, CA, USA) and Alexa Fluor-488 goat anti-rabbit and anti-mouse IgG (Invitrogen, CA, USA).

2. Breast cancer cell culture

The human breast cancer cell lines SKBR3, BT474 (American Type Culture Collection, MD, USA) and JIMT-1 (DSMZ GmbH, Germany) cells were cultured in DMEM

or RPMI1640 (Gibco, MD, USA) containing 10% fetal bovine serum (FBS) and streptomycin-penicillin (100 U/ml). The cells were incubated at 37 in an atmosphere of 5% CO2. All the cell lines were authenticated using short tandem repeat (STR) profiling (Macrogen Inc., Seoul, Republic of Korea).

3. Cell viability assay

The cell viability was determined using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay [MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (Promega, Madison, WI, USA) according to the manufacturer's instructions. The quantity of the formazan product was measured via the absorbance at 490 nm with a SpectraMax Plus 384 microplate analyzer (Molecular Devices, CA, USA)

4. Cell cycle analysis and annexin V/PI assay

The cells were harvested and fixed with 95% ethanol containing 0.5% Tween-20 for 24 hours. After washing with phosphate buffered saline (PBS), the cells were incubated with PI (50 μg/ml) and RNase (50 μg/ml), for 30 minutes. The percentage of cells in each phase of the cell cycle was determined and the cells belonging to the sub-G1 population were considered apoptotic. For the Annexin V/PI assay, a FITC-conjugated Annexin V apoptosis detection kit (BD Biosciences, NJ, USA) was used according to the manufacturer's protocol.

The stained cells were analyzed using flow cytometry with a BD LSRFortessa™ X-20 Cell Analyzer (BD Biosciences, NJ, USA).

5. Immunoblot analysis

The cells were solubilized in a lysis buffer [30 mM NaCl, 0.5% Triton X-100, and 50 mM Tris-HCl (pH 7.4)] that contained phosphatase and protease inhibitor cocktail tablets. The supernatant was collected after centrifugation (14,000 g, 4 °C, 20 min) and the protein concentrations measured using a Bradford protein assay kit (Bio-Rad Laboratories, CA, USA). Equal quantities of the protein (30 μg) were subjected to SDS-PAGE and electrotransferred onto a nitrocellulose membrane (GE Healthcare Life Sciences, Buckinghamshire, UK). The membranes were incubated overnight at 4 . The primary antibodies were diluted in 5% bovine serum albumin (BSA) [HER2 (1:2000), p-HER2 (1:2000), HER3 (1:2000), p-HER3 (1:2000), EGFR (1:3000), p-EGFR (1:2000), MEK (1:2000), p-MEK (1:2000), ERK (1:2000), p-ERK (1:2000), Akt (1:2000), p- Akt (1:2000), PARP (1:2000), cleaved-caspase-3 (1:1000), cleaved-caspase-7 (1:1000), cleaved-caspase-8 (1:1000), and GAPDH (1:3000)], followed by incubation with HRP-conjugated anti-rabbit and mouse IgGs (1:2000). The signal intensity was detected using an Enhanced Chemiluminescence Kit (Thermo Scientific Inc., IL, USA) and x-ray film (Agfa Healthcare, Mortsel, Belgium).

6. Immunocytochemistry

The cells were fixed with 4% paraformaldehyde (BD Biosciences, NJ, USA) on 8-well chamber slides, washed with PBS, and incubated with 0.2% Triton X-100 for 10 minutes. The cells were incubated overnight at 4 with the primary antibodies in antibody-diluent (Dako, Glostrup, Denmark). For the secondary antibody reactions, Alexa Fluor®-488 conjugated secondary antibodies (Invitrogen, CA, USA) were used, then the cells were mounted using the ProLong Gold Antifade Reagent with DAPI (Life Technologies, CA, USA). The images were acquired using a Carl Zeiss confocal microscope (Weimar, Germany), and the intensity of the images was analyzed using the intensity profiling tool.

7. ALDEFLUOR-positivity assay and CD24/44 staining

An ALDEFLUOR assay kit (STEMCELLTechnology, BC, Canada) was used to evaluate the ALDH1 activity according to the manufacturer's protocol. The cells were incubated at 37 for 45 minutes in an ALDEFLUOR assay buffer containing the ALDH protein substrate, BODIPY- aminoacetaldehyde (BAAA, 1μM / 0.5 x 10^6 cells). As a specific inhibitor of ALDH1, 50mM of diethylamino-benzaldehyde (DEAB) was used to define the ALDEFLUOR-positive population. For the CD24/44 staining, the cells were stained with FITC- and PE-conjugated anti-mouse IgG, and either FITC-conjugated anti-CD24 or PE-conjugated anti-CD44 (BD Biosciences, NJ, USA). The ALDEFLUOR- and CD24/44-stained

cells were analyzed using a BD LSRFortessa™ X-20 Cell Analyzer (BD Biosciences, NJ, USA).

8. Mammosphere formation assay

For the *in vitro* mammosphere-forming assay, the cells were plated in ultra-low attachment dishes (Corning, NY, USA) and the HuMEC basal serum-free medium (Gibco, MD, USA), supplemented with B27 (1:50, Invitrogen, CA, USA), 20 ng/ml basic fibroblast growth factor (bFGF, sigma-Aldrich, MO, USA), 20 ng/ml human epidermal growth factor (hEGF, sigma-Aldrich, MO, USA), 4 μg/ml heparin, 1% antibiotic-antimycotic, and 15 μg/ml gentamycin at 37 , in an atmosphere of 5% CO_2. The numbers and volumes of the mammospheres were measured under an Olympus CKX53 inverted microscope.

9. Statistical analysis

All the statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, CA, USA). The results were presented as mean values ± the standard error of mean (SEM) of at least three independent experiments. The data were analyzed using the Student's t-test, one-way ANOVA, or two-way ANOVA, as appropriate, and multiple comparisons were made using the Bonferroni's post hoc test. Statistical significance was defined at $P < 0.05$.

Results

1. Aprepitant reduces cell viability in HER2-positive breast cancer cells in vitro

The cytotoxic effects of aprepitant (Figure 1A) in HER2-positive breast cancer cells were evaluated using the trastuzumab-sensitive cell lines, SKBR3 and BT474, and the trastuzumab-resistant cell line, JIMT-1. These cells were treated with various concentrations (0.01–80 μM) of aprepitant for 72 hours. The MTS assays revealed that cell viability showed a significant decrease in response to aprepitant in a dose-dependent manner, and the IC_{50} values of aprepitant were 12.29 μM, 26.50 μM, and 10.01 μM in SKBR3, BT474, and JIMT-1 cells, respectively (Figure 1B).

To investigate the effect of aprepitant on cell morphology in HER2-positive breast cancer cell lines, SKBR3, BT474, and JIMT-1 cells were treated with 10 μM and 20 μM of aprepitant for 72 hours and phase contrast microscopic examinations were performed. Aprepitant treatment was found to induce cytotoxicity in all of the three cell lines by changes in the cell morphology such as cytoplasmic shrinkage and the presence of round free-floating single cells (Figure 2).

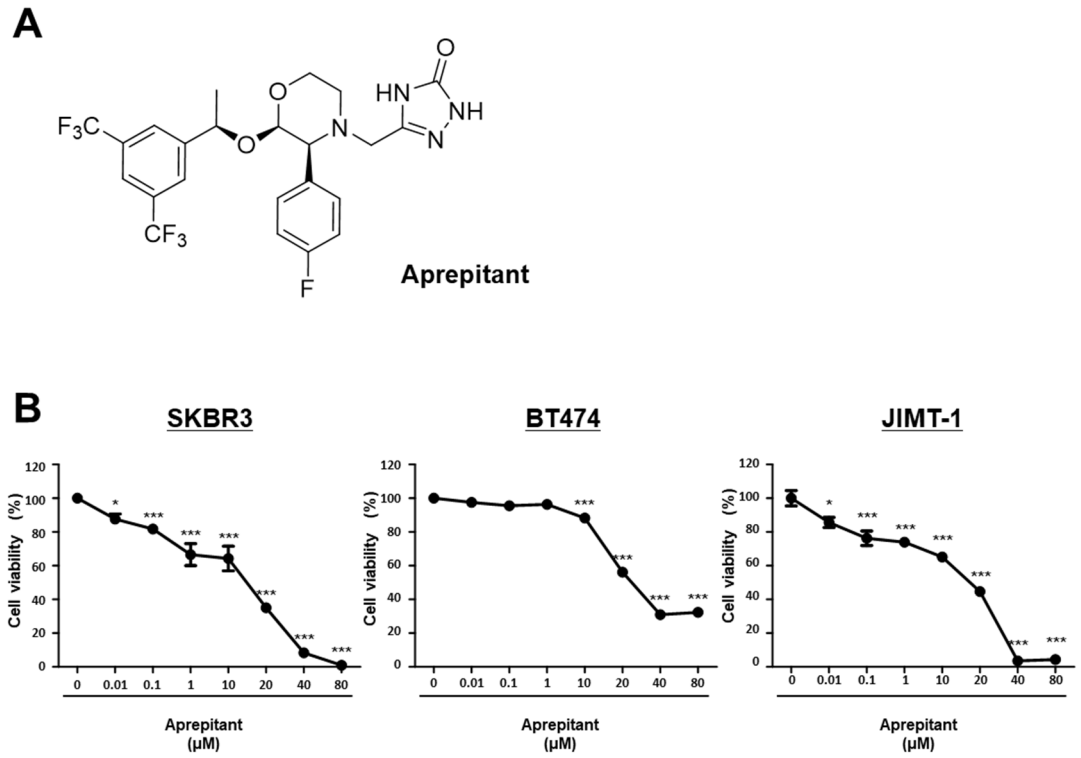

Figure 1. Aprepitant suppresses cell viability in trastuzumab-sensitive and -resistant HER2-positive breast cancer cells.

(A) Chemical structural of aprepitant. **(B)** Trastuzumab-sensitive (SKBR3 and BT474) and -resistant (JIMT-1) HER2-positive breast cancer cells were treated with various concentrations of aprepitant (0.01–80 μM) during the indicated periods for 72 h. Cell viability was determined using MTS assays (* $P < 0.05$, *** $P < 0.001$, versus DMSO control). The results are presented as means ± SEMs of at least three independent experiments and analyzed using one-way ANOVA tests followed by Bonferroni post hoc tests.

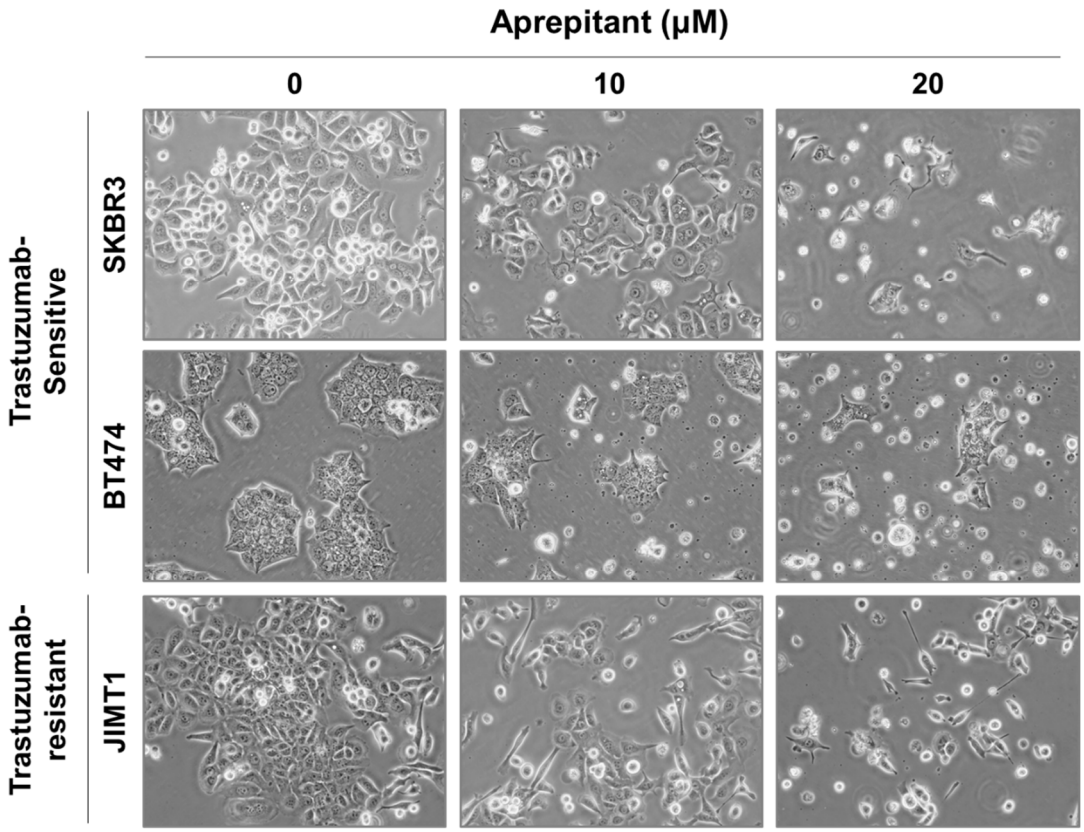

Figure 2. Aprepitant induced changes in cellular morphology.

Changes in cellular morphology in SKBR3, BT474, and JIMT-1 cells after aprepitant (0–20 μM) treatment for 72 h as seen through phase contrast microscopy. Images are shown at magnification (× 200).

2. Aprepitant induces apoptosis as evidenced by increased Sub-G1 accumulation and Annexin V-positive cell populations

Next, we examined the effect of aprepitant on apoptosis in trastuzumab-sensitive BT474 and -resistant JIMT1 HER2-positive breast cancer cells. The cells were treated with aprepitant (10 and 20 μM) for 72 hours, and the deoxyribonucleic acid (DNA) fragmentation during apoptosis in the cells was measured using Sub-G1 analysis with flow cytometry. Aprepitant significantly increased Sub-G1 accumulation in both cells (Figure 3A). The quantitative graphs of the Sub-G1 population are presented in Figure 3B. Apoptosis assays with Annexin V/PI staining were further performed to quantify the apoptosis of the different cell populations (live, early and late apoptosis, and necrosis). Both the early and late apoptotic cells showed a significant increase in the presence of aprepitant (20 μM, 72 hours), both in the BT474 and JIMT-1 cells (Figure 4A). The quantitative graphs of Annexin V-positive cells are shown in Figure 4B.

3. Aprepitant induces apoptosis in HER2-positive breast cancer cells, mediated by caspase activation

The poly (ADP-ribose) polymerase (PARP) is involved in the regulation of cellular processes such as genomic stability, DNA repair, and cell survival as well as programmed cell

death [9]. PARP is a major substrate of caspases and its cleaved-form is considered to be a hallmark of apoptosis [10]. To determine the expression of apoptosis-related factors in the presence or absence of aprepitant in BT474 and JIMT-1 cells (10 and 20 μM, 72 hours), immunoblotting assays were performed. Aprepitant treatment resulted in marked increases in the cleaved caspases -3, -7, and -8 protein contents and an increased PARP cleavage in both the BT474 and JIMT-1 cells (Figure 5).

4. Aprepitant markedly suppresses the MEK/ERK phosphorylation of HER2-positive breast cancer cells

Given that SP interacts with NK1R to activate members of the mitogen-activated protein kinase (MAPK) cascade including the extracellular signal-regulated kinases 1 and 2 (ERK1/2) [11] which are key signaling pathways involving cell proliferation, survival, and differentiation, we evaluated the changes in protein levels including SP, NK1R, MEK, phospho-MEK (Ser217/221), ERK, and phospho-ERK (Thr202/Tyr204) in HER2-positive breast cancer cells after aprepitant treatment (10–20 μM, 72 hours). A western blot analysis revealed that aprepitant downregulated the protein content of SP and NK1R considerably, concomitant with a reduction of MEK/ERK expression and their phosphorylation in trastuzumab-sensitive and -resistant cells (Figure 6).

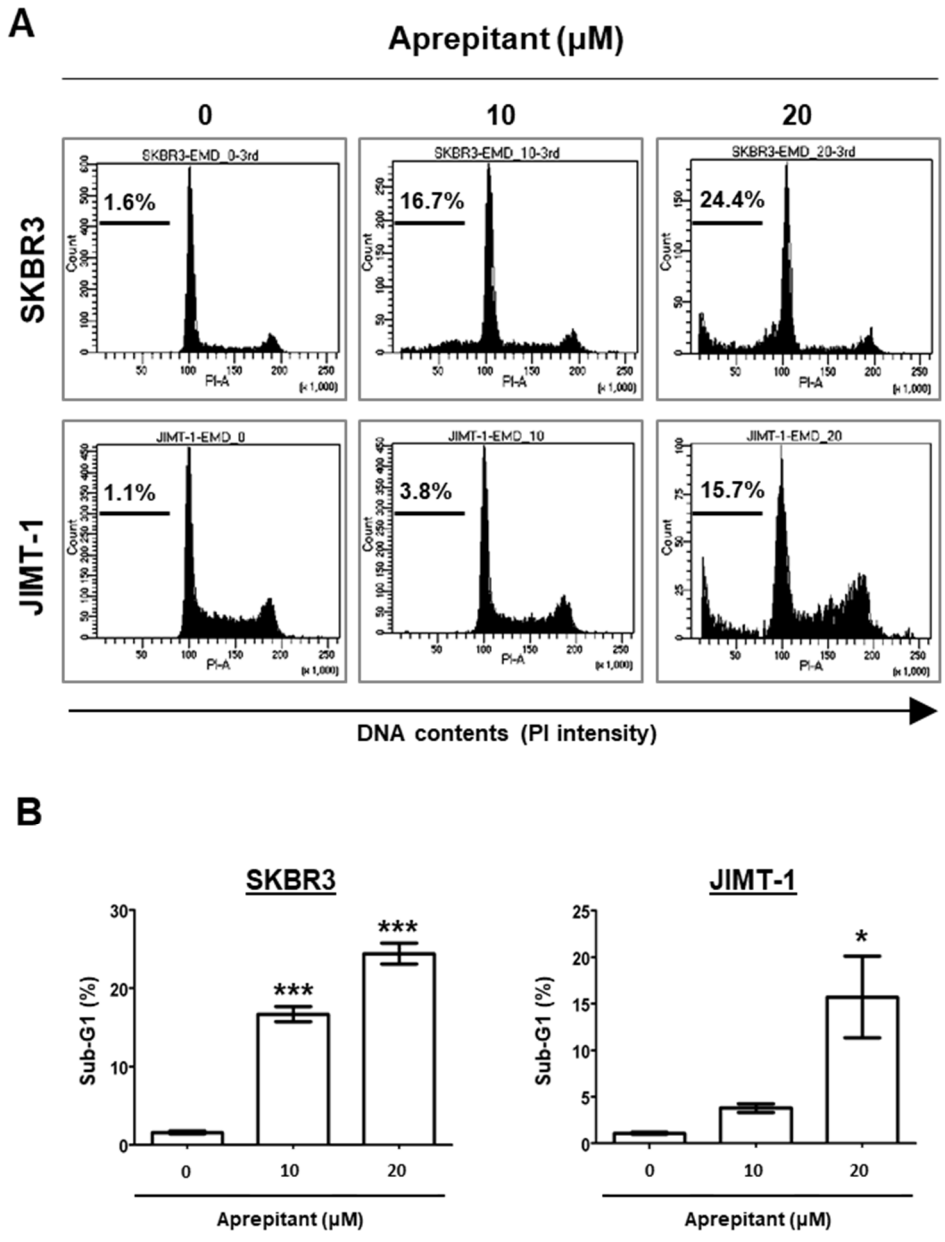

Figure 3. Aprepitant resulted in a significant induction of Sub-G1 accumulation.

(A) Sub-G1 analysis was assessed using flow cytometry. Aprepitant (10–20 μM, 48 h) induced apoptosis significantly in both trastuzumab-sensitive SKBR3 and trastuzumab-resistant JIMT-1 cells, as evidenced by an increased Sub-G1 population. **(B)** The quantitative graphs represent the Sub-G1 population (* $P < 0.05$, *** $P < 0.001$). The results are presented as means ± SEMs of at least three independent experiments and analyzed using one-way ANOVA tests followed by Bonferroni post hoc tests.

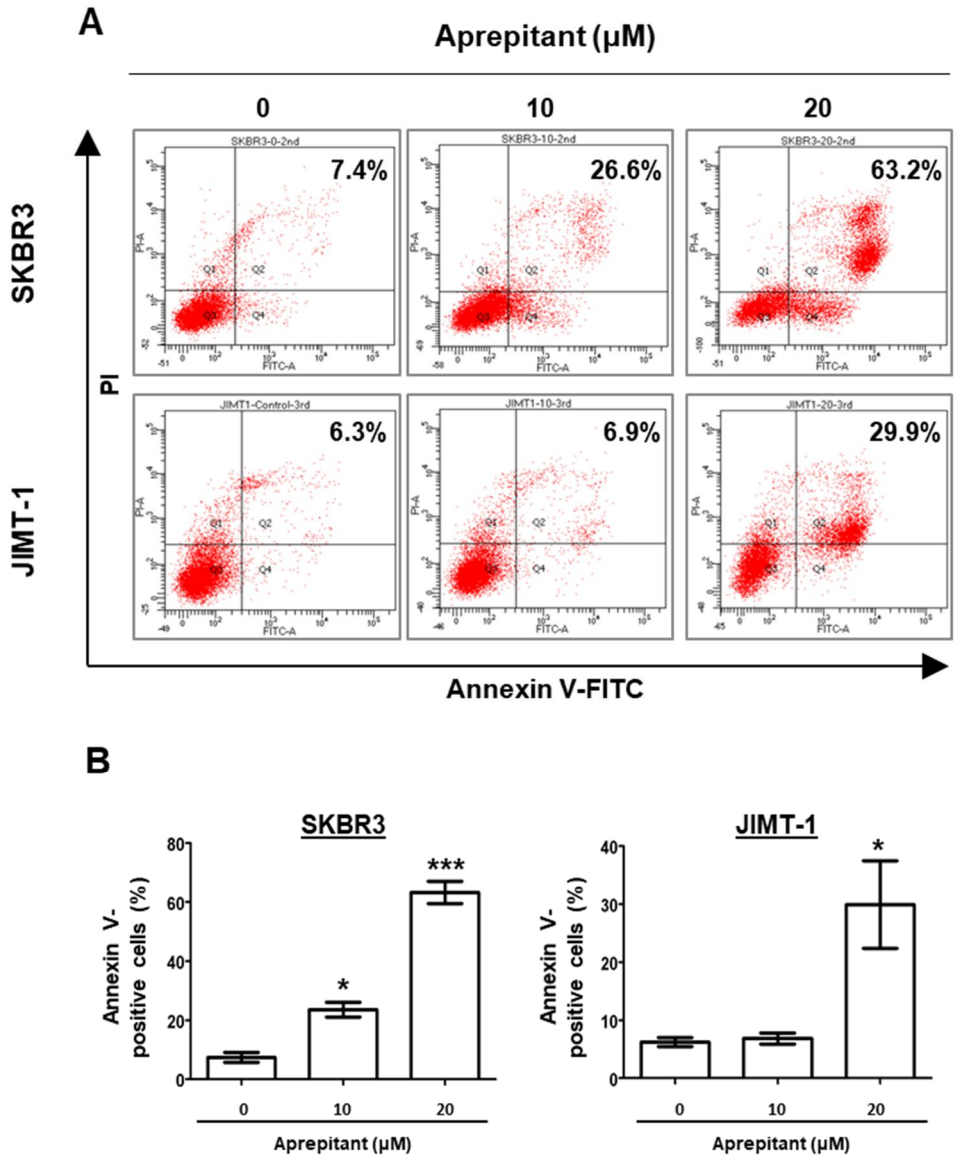

Figure 4. Aprepitant induced early and late apoptosis in HER2-positive breast cancer cells.

(A) The SKBR3 and JIMT-1 cells were treated with aprepitant (0–20 μM) for 72 h and Annexin V/PI staining was analyzed using flow cytometry. **(B)** The percentage of annexin V/PI-positive cells (early and late apoptotic cells) was quantified (* $P < 0.05$, *** $P < 0.001$). The results are presented as means ± SEMs of at least three independent experiments and analyzed using one-way ANOVA tests followed by Bonferroni post hoc tests.

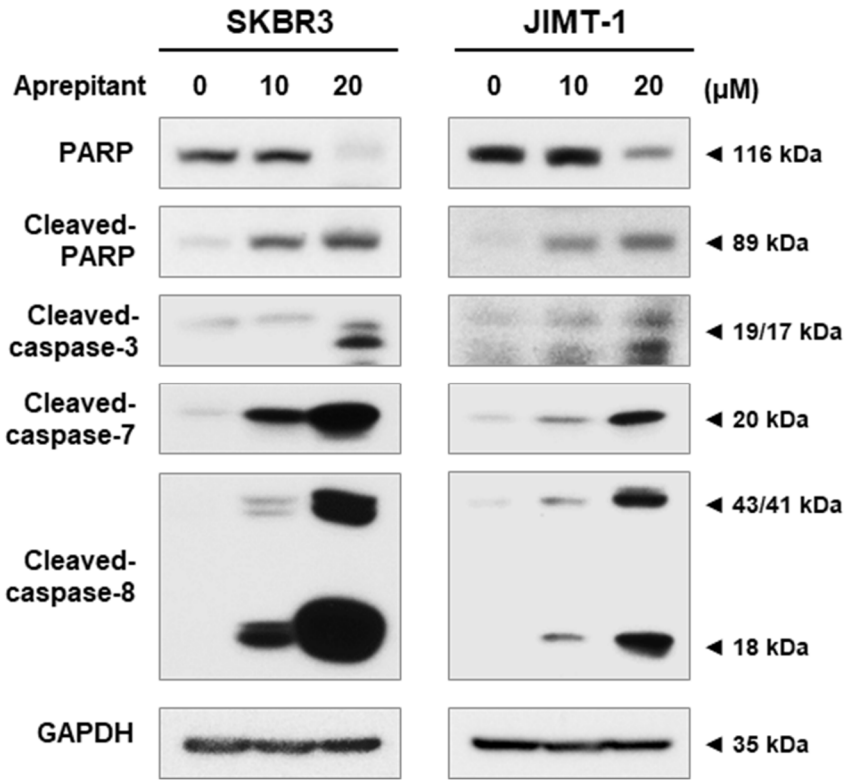

Figure 5. Aprepitant-induced apoptosis was mediated by activation of caspase-3/-7/-8.

The SKBR3 and JIMT-1 cells were treated with aprepitant (0–20 μM) for 72 h and the protein expression levels of apoptosis-related factors including PARP, cleaved-PARP, cleaved-caspase-3, cleaved-caspase-7 and cleaved-caspase-8 were determined using western blot analyses. GAPDH was used as a loading control.

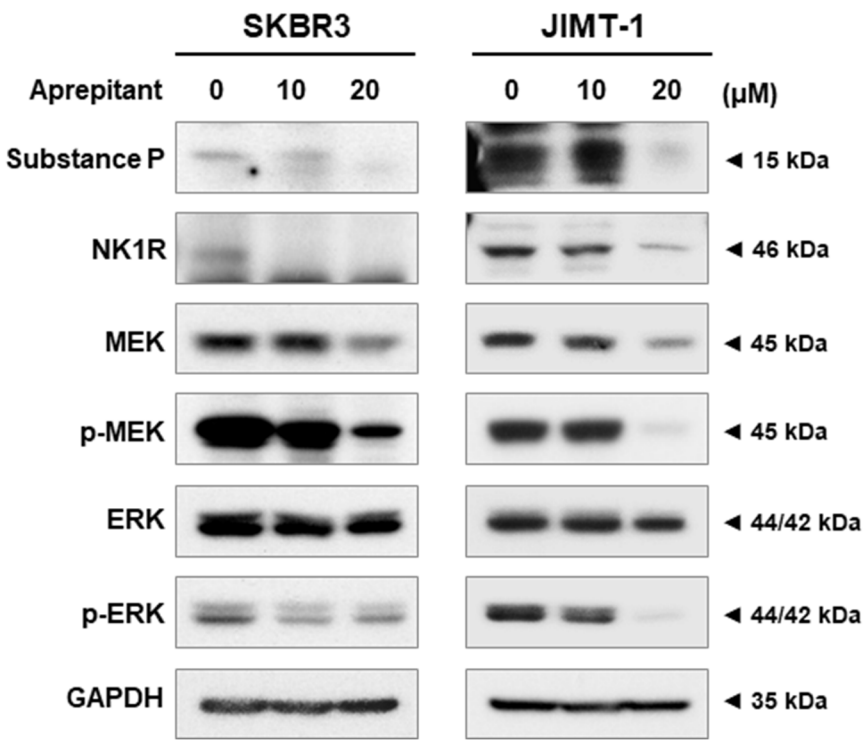

Figure 6. Aprepitant inhibits SP-NK1R signaling, accompanied with suppression of MEK/ERK phosphorylation.

The SKBR3 and JIMT-1 cells were treated with aprepitant (0–20 μM) for 72 h and the protein expression levels of Substance P (SP), NK1R, MEK, phospho-MEK (Ser217/221), ERK, and phospho-ERK (Thr202/Tyr204) were determined using western blot analyses. GAPDH was used as a loading control.

5. Aprepitant affects HER2 signaling via downregulating HER2/HER3 and truncated p95HER2

The HER2 activity is ligand independent and the HER2-HER3 hetero-dimerization has been shown to be the most potent oncopair in the activation of tumor cell proliferation [12]. In general, HER2 refers to the full-length form of p185 HER2, and trastuzumab binds its extracellular domain. However, the metalloproteases cleave the extracellular domain of HER2, generating a truncated receptor (p95 HER2) with tyrosine kinase activity that trastuzumab cannot bind to [13].

Next, we examined whether aprepitant affects the HER2 signaling pathway in HER2-positive breast cells. Immunoblotting assays showed that the protein expression of HER2, HER3, and even p95HER2 showed a marked reduction in the SKBR3 cells following aprepitant treatment (0–20 μM, 72 hours). Aprepitant also suppressed the phosphorylation of HER2 (Tyr1221/1222) and HER3 (Tyr1289), and subsequently reduced Akt expression in the SKBR3 cells. Considerable downregulation of p95HER2, HER3, and phospho-HER3, as well as a marked reduction of Akt were also observed with aprepitant treatment (0–20 μM, 72 hours) in trastuzumab-resistant JIMT-1 cells; however, no change in the HER2 or phospho-HER2 expressions were noted (Figure 7).

We also performed immunostaining analyses to further confirm the inhibitory effect of aprepitant on the expression of full-length HER2 and intracellular (ICD) HER2 in trastuzumab-sensitive BT474 cells. After exposure to aprepitant (20 μM, 24 hours), both the cytoplasmic membrane HER2 (Figure 8A) and ICD-HER2 expression (Figure 8B) were markedly suppressed, when compared to their control counterparts. Taken together, these results suggested that aprepitant effectively targets HER2 signaling in both trastuzumab-sensitive and -resistant HER2-positive breast cancer cells.

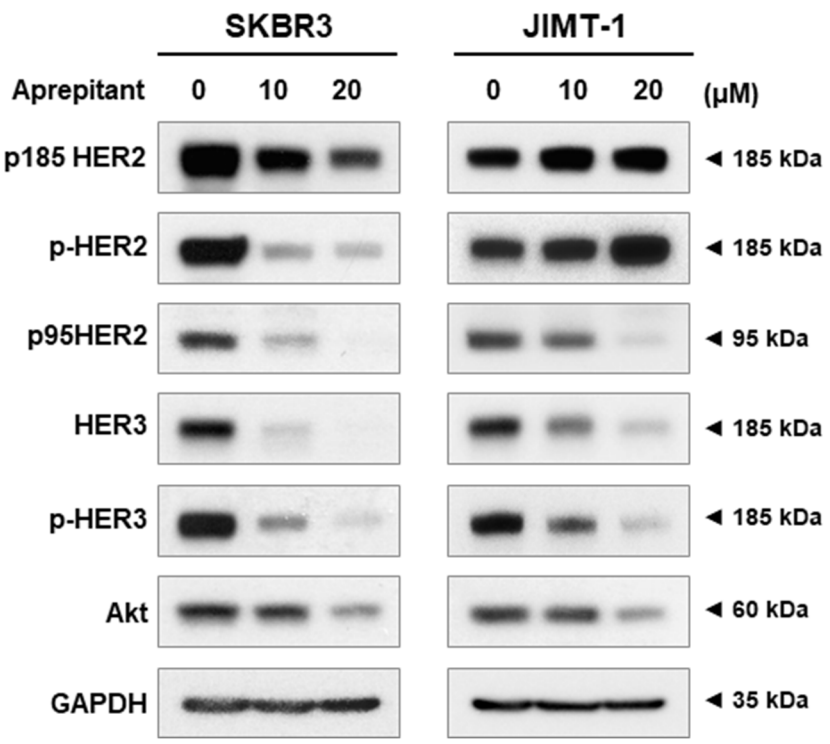

Figure 7. Aprepitant downregulates HER2/HER3 and truncated p95HER2.

The SKBR3 and JIMT-1 cells were treated with aprepitant (0–20 μM) for 72 h and the protein expression levels of full-length p185HER2, amino-terminal-truncated p95HER2, phospho-HER2 (Tyr1221/1222), HER3, phospho-HER3 (Tyr1289), and Akt were determined using western blot analyses. GAPDH was used as a loading control.

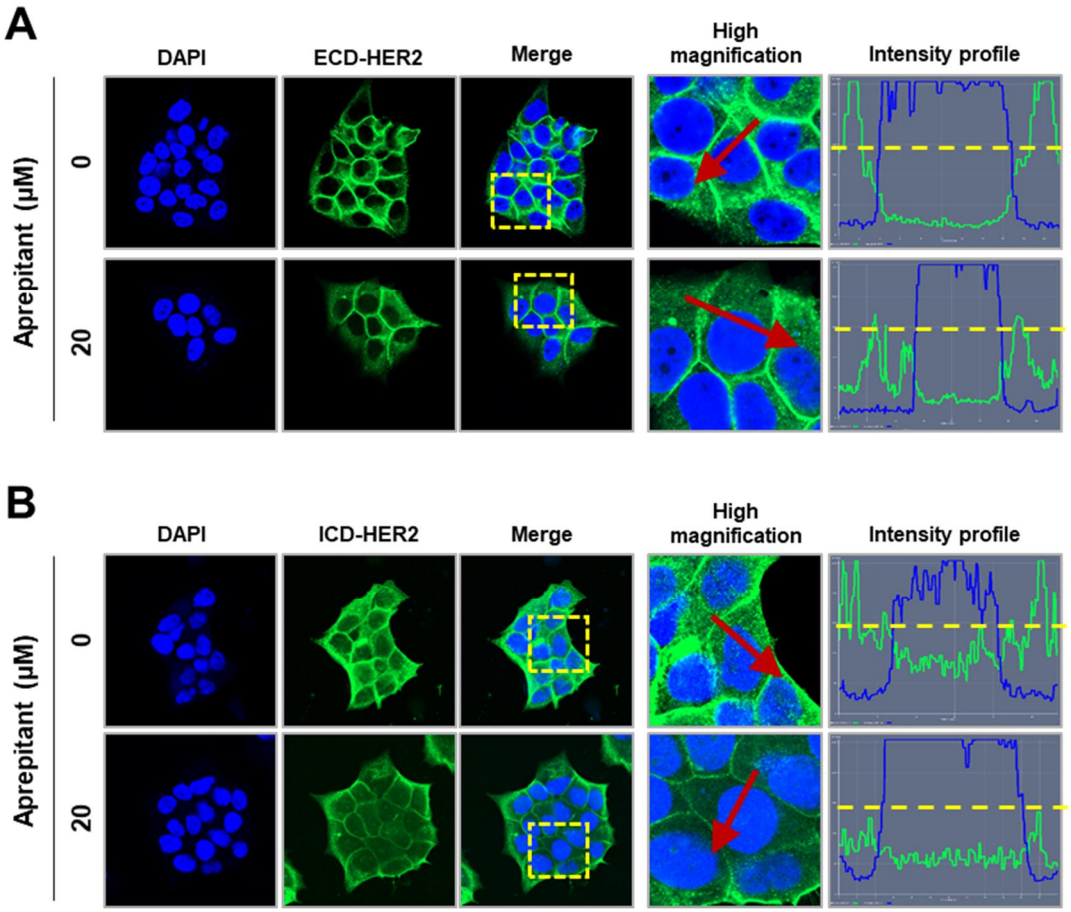

Figure 8. Aprepitant downregulates both full-length HER2 and intracellular domain HER2 expression.

The BT474 cells were immunostained for (A) full-length HER2 and (B) ICD-HER2 (both 1:100, green color) with DAPI (nuclear, blue) after exposure to aprepitant (20 μM) and for 24 h. The images were acquired using Carl Zeiss confocal microscopy at × 500 magnification. Intensities of (A) cytoplasmic membrane HER2 and (B) ICD-HER2 (green) are represented in arbitrary units as defined by the software of the intensity profiling tool.

6. Aprepitant targets cancer stem-like properties in HER2-positive breast cancer cells

Since mounting evidence has suggested that cancer stem cells (CSC) contribute to development of treatment resistance and metastases [14], we investigated whether aprepitant affected the BCSC-like properties in HER2-positive breast cancer. The BCSC-like characteristics were evaluated using ALDH1 activity, $CD44^{high}/CD24^{low}$ ($CD44^{+}/CD24^{-}$) phenotypes, and the *in vitro* mammosphere-forming ability.

Previous studies demonstrated that ALDH1 activity plays a pivotal role in HER2-mediated tumorigenesis and its expression is highly elevated in HER2-positive breast cancers [15, 16]. In this study, the ALDEFLUOR-positivity assay revealed that ALDH1 activity shown to have a significantly diminished response to aprepitant (10–20 μM, 72 hours) in both the SKBR3 and JIMT-1 cells (Figure 9A-B). There was also no significant change in the proportion of ALDEFLUOR-positive cells after exposure to aprepitant (20 μM, 72 h) on HER2-negative MDA-MB-231 breast cancer cells (Supplemental figure 1). Moreover, it is known that the tumorigenic cancer cells can be distinguished based on cell surface marker expression, and the $CD44^{+}/CD24^{-}$ phenotype represents CSC-like properties in breast cancers [17]. Furthermore, CD24/44 staining was performed only in the JIMT-1 cells in the present study, given that trastuzumab-sensitive SKBR3 and BT474 cell lines harbored limited numbers of the $CD44^{+}/CD24^{-}$ population at approximately, 0.9% and 0.03%, respectively [18]. As

shown in Figure 10A, trastuzumab resistant JIMT-1 cells exhibited substantially higher levels of $CD44^{+}/CD24^{-}$ cells, and this stem-like population showed a significant reduction after treatment with 20 μM of aprepitant for 72 hours (Figure 10B).

Under anchorage-independent serum-free culture conditions, the BT474 cells showed an ability to form mammospheres which possess highly enriched BCSC-like populations with self-renewal capacity [19]. After a 4-day of culture in serum-free conditions in ultra-low attachment plates, the control mammospheres exhibited regular three-dimensional spheres; however, this sphere-forming ability was suppressed dramatically by aprepitant (10–20 μM) (Figure 11A), as evidenced by the number and volume of mammospheres derived after treatment (Figure 11B).

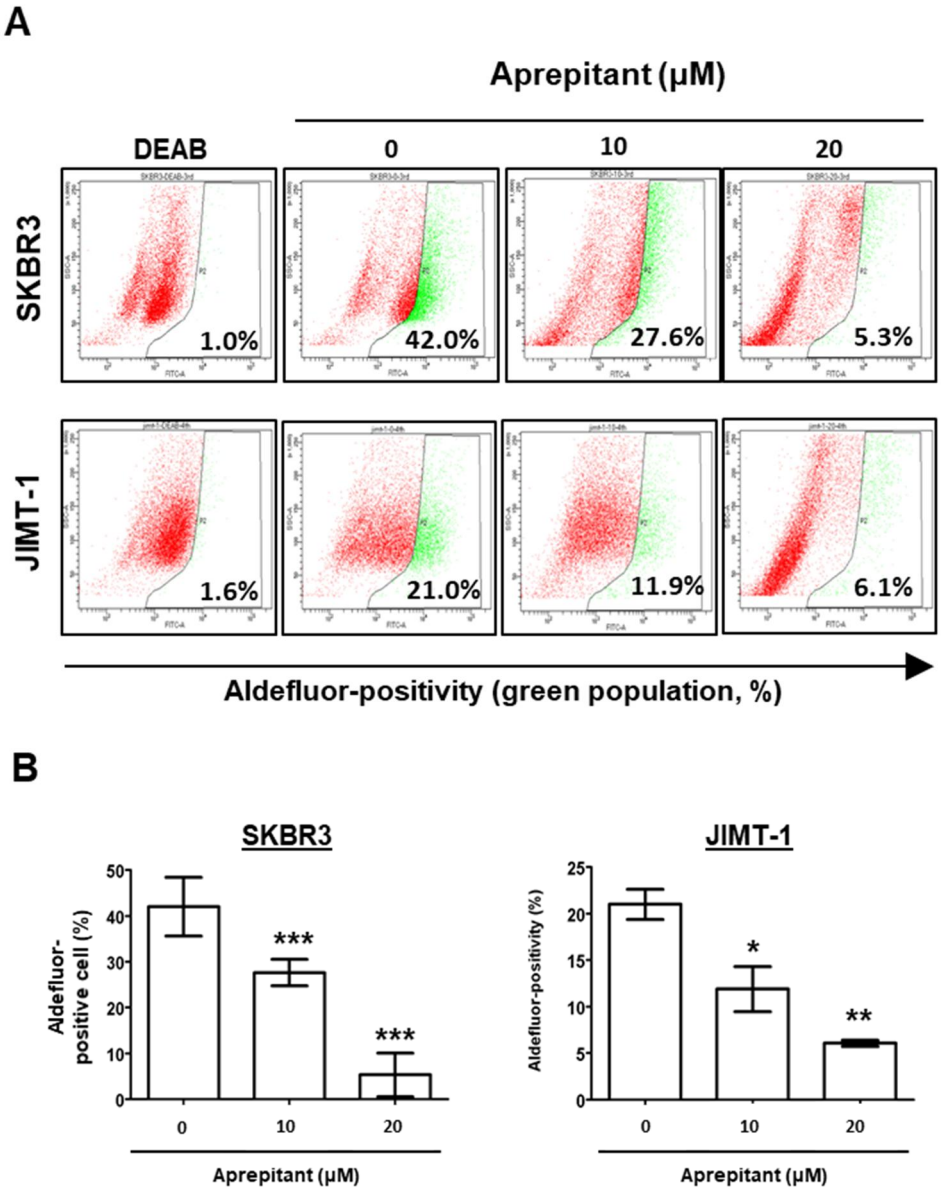

Figure 9. Aprepitant targets BCSC via suppression of ALDH1 activity.

(A) After exposure to aprepitant (10–20 μM, 72 h), ALDEFLUOR positivity was abolished in the SKBR3 and JIMT-1 cells. The ALDEFLUOR-positive cells were analyzed using flow cytometry. As a specific inhibitor of ALDH1, 50 mM diethylamino-benzaldehyde (DEAB) was used for defining the ALDEFLUOR-positive population. **(B)** The quantitative graphs of ALDEFLUOR-positive cells (* $P < 0.05$, ** $P < 0.01$, *** $p < 0.001$). The results are presented as means ± SEMs of at least three independent experiments and analyzed using one-way ANOVA tests followed by Bonferroni post hoc tests.

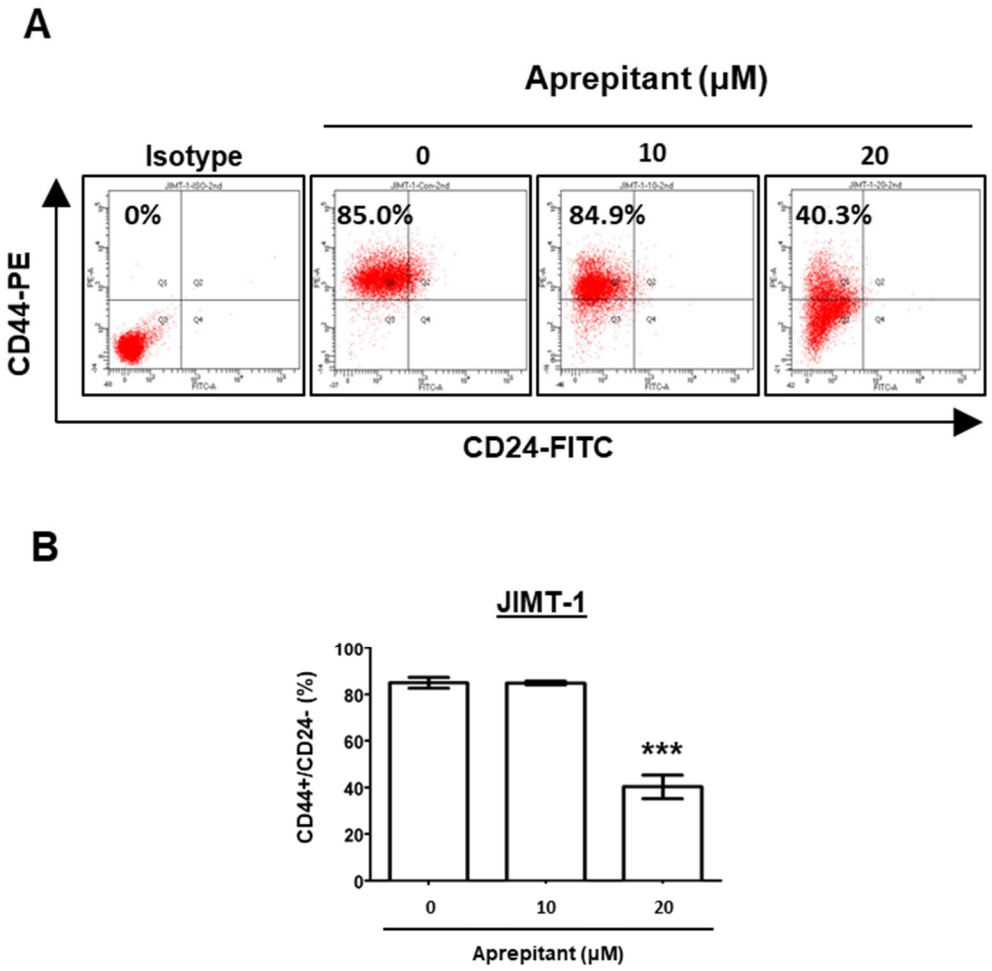

Figure 10. Aprepitant decreased CD44$^+$/CD24$^-$ -stem-like population in trastuzumab-resistant JIMT-1 cells.

(A) The CD44$^+$/CD24$^-$ population of JIMT-1 cells showed a marked decrease in the presence of aprepitant (10–20 μM, 72 h). The CD44$^+$/CD24$^-$ population was analyzed using flow cytometry. Isotype- control was used to define the CD44$^+$/CD24$^-$ population. **(B)** The quantitative graphs of the CD44$^+$/CD24$^-$ population (*** $P < 0.001$). The results are presented as means ± SEMs of at least three independent experiments and analyzed using one-way ANOVA tests followed by Bonferroni post hoc tests.

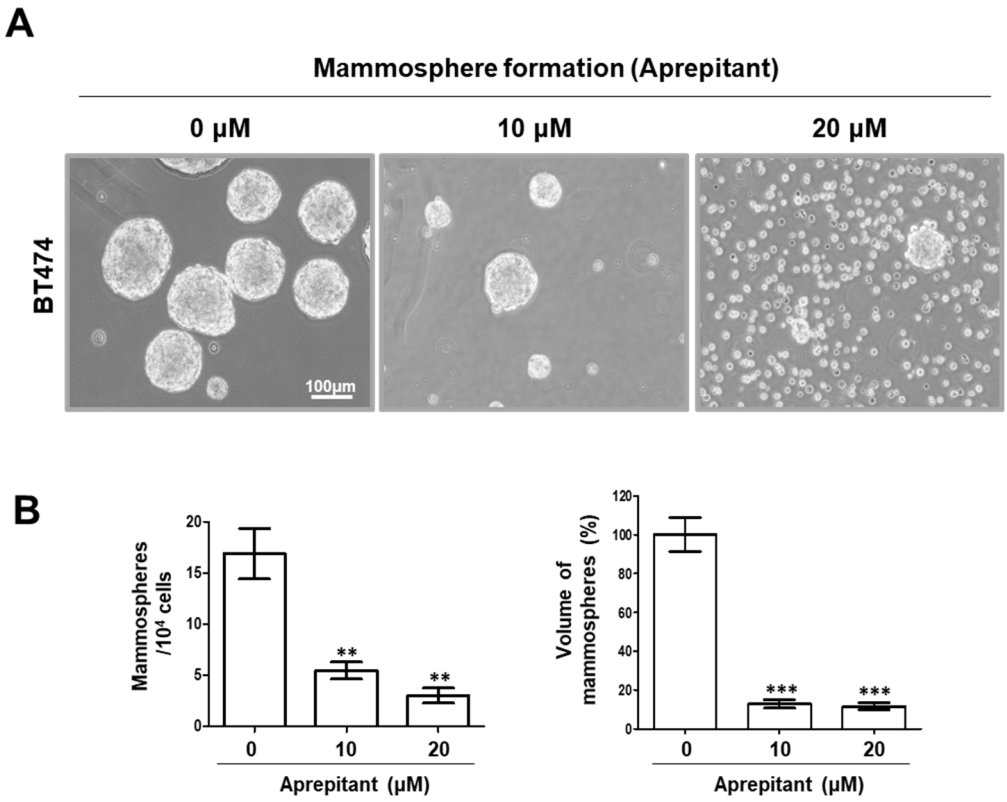

Figure 11. Aprepitant significantly suppressed the mammosphere forming-ability in HER2-positive breast cancer cells.

(A) The BT474 cells (3×10^5) were cultured in ultralow attachment plates in the presence or absence of aprepitant (10 and 20 μM) for four days. The mammosphere forming ability showed dramatic suppression after the aprepitant challenge. **(B)** The number and volume of the mammospheres derived from the BT474 cells were quantified (** $P < 0.01$, *** $P < 0.001$).

7. Aprepitant overcomes trastuzumab-resistance in HER2-positive breast cancer cells

When treated with trastuzumab (0–300 μg/mL, 72 h), the viability of the BT474 cells showed a significant decrease at concentrations above 100 μg/mL, whereas the JIMT-1 cells were not sensitive to trastuzumab (Figure 12A). Next, using trastuzumab-sensitive BT474 cells and trastuzumab-resistant JIMT-1 cells, we examined whether aprepitant could sensitize cells to trastuzumab. The cells were treated with trastuzumab (0–100 μg/ mL) in the presence or absence of aprepitant (20 μM) for 72 hours and the cell viability was analyzed. It was observed that the combination treatment of trastuzumab and aprepitant showed an additional inhibitory effect on cell viability in the BT474 cells. However, in trastuzumab-resistant JIMT-1 cells, no synergistic inhibitory effect was exerted, compared with the individual treatment with aprepitant (Figure 12B). Our findings suggested that aprepitant could overcome trastuzumab resistance on its own, and it could be a new alternative drug for patients who experienced treatment failure with trastuzumab.

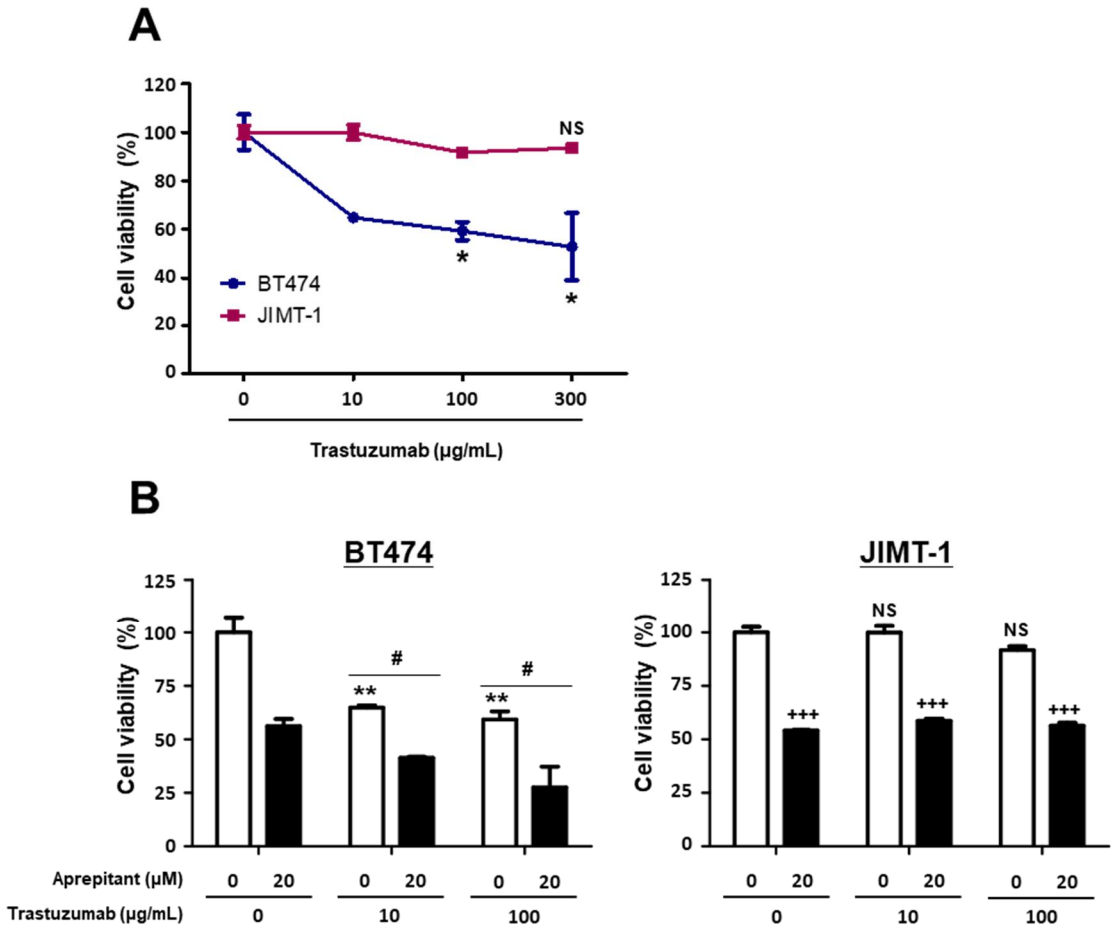

Figure 12. The combined treatment of aprepitant and trastuzumab did not exert a synergistic inhibitory effect on the viability of HER2-positive breast cancer cells.

(A) The effect of trastuzumab (0–300 μg/mL, 72 h) on the viability of the BT474 and JIMT-1 cells was determined using MTS assays (* $P < 0.05$; NS, no significance). The trastuzumab-sensitive BT474 cells exhibited sensitivity to trastuzumab, whereas the JIMT-1 cells showed no significant response to trastuzumab. **(B)** The BT474 and JIMT-1 cells were treated with the indicated concentrations of aprepitant (0–20 μM) and trastuzumab (0–100 μg/mL) for 72 hours. The cell viability was analyzed using MTS assays. The results are expressed as means ± SEMs (n = 3 independent replicates) and analyzed using two-way ANOVA tests followed by Bonferroni post hoc tests [** $P < 0.01$; NS, no significance (distilled water control vs. trastuzumab); # $P < 0.05$ (trastuzumab only vs. trastuzumab + aprepitant); +++ $P < 0.001$ (DMSO control vs. aprepitant)]

Discussion

Drug repurposing, also known as drug repositioning, re-tasking, or re-profiling, is a strategy for identifying and developing new uses of approved or investigational drugs that are outside the scope of the original indication [20]. The use of de-risked compounds and the potential to reduce developmental time and cost makes this strategy an increasingly attractive proposition [21]. Based on the considerable evidence of previous studies that demonstrated the oncogenic properties of the SP-NK1R system [22-24] and their associations with breast cancer tumorigenesis via their effect on multiple processes including HER2 activation, we explored the impact of aprepitant, a NK1R antagonist, on cell viability, apoptosis, HER2 signaling pathways and the cancer stem cell-like properties in trastuzumab-sensitive and -resistant HER2-positive breast cancer cells, *in vitro*.

The usual dose of aprepitant as an anti-emetic for CINV is 125 mg or 80 mg, as part of 3-day regimen (125-80-80 mg) that includes 5-HT_3 antagonists and dexamethasone. The maximum plasma concentration at the indicated dose is approximately 1500 ng/ml [25], which corresponds to 2.8 μM. Several clinical studies have demonstrated the safety and tolerability of aprepitant even with prolonged use or at higher doses (375 mg/day for two weeks; 80 mg/day initially followed by an increase to 125 mg/every third day for seven months; 160 mg/day for 45 days; 300 mg/day for 45 days) [26-29]. Moreover, no toxicity was observed up

to a concentration of 40 times the clinical exposure when tested in dogs [30], and the carcinogenic doses of aprepitant reported from animal studies were 125–2000 mg/kg/day [6]. In the present study, aprepitant concentrations of 10–20 μM were selected based on the IC_{50} values derived from MTS assays performed on the three different HER2-positive breast cancer cell lines (12.29 μM, 26.50 μM, and 10.01 μM, in SKBR3, BT 474, and JIMT-1 cells, respectively) and we examined the anti-tumor effect of aprepitant at these concentrations, which were much less than the concentrations known to cause toxicity.

We observed that exposure to 10–20 μM of aprepitant for 72 hours suppressed cell viability significantly and induced caspase-mediated cell death. The activation of caspase-3, -7, and -8 indicated that both the intrinsic and extrinsic pathways of apoptosis were involved in aprepitant-induced cell death [31]. Furthermore, it is well known that the activation of ERK1/2 inhibits apoptosis and promotes cell survival suppressing the functions of pro-apoptotic proteins including caspase, enhancing the activity of anti-apoptotic molecules, and increasing the expression and activity of DNA repair proteins [32]. The activity of ERK1/2 is positively regulated by MEK1/2 (MAPKK) phosphorylation, and it has been demonstrated that SP-NK1R binding triggers the Ras/Raf/MAPK signaling cascade [23]. In this context, we found that aprepitant was effective in reducing the expression and phosphorylation of MEK and ERK in both the trastuzumab-sensitive and -resistant breast cancer cells.

It has been demonstrated that SP-NK1R autocrine signaling contributed to persistent transmodulation of EGFR and HER2 prompting tumor progression and drug resistance in breast cancer cells, and conversely SP abrogation decreased their steady states [8, 33]. A considerable downregulation of HER2 was also observed in SKBR3 cells after aprepitant treatment; however, this was not observed in JIMT-1 cells, a trastuzumab-resistant cell line. Instead, we found that aprepitant decreased the expression and activity of HER3 notably, in both the SKBR3 and JIMT-1 cells. As the most potent signaling pair within the ErbB family [34], HER2/HER3 heterodimerization weakens the power of trastuzumab-based HER2 target therapy, given that trastuzumab is unable to block the dimerization interface of HER2 [35]. Moreover, the compensatory crosstalk among receptors within the HER family, like the HER1/HER3 heterodimers, can initiate MAPK and PI3K signaling even in the presence of trastuzumab [36, 37]. This is one of the well-known mechanisms explaining trastuzumab resistance. Therefore, the downregulation of HER3 induced by aprepitant may have played a major role in overcoming trastuzumab resistance. In addition, aprepitant downregulated truncated intracellular HER2, which retained HER2 kinase activity but lacked the extracellular trastuzumab-binding domain in breast cancer cells [38]. Although the presence of truncated-p95HER2 was thought to be insufficient to explain the drug resistance in the JIMT-1 model due to its minimal expression [39], we observed an apparent decrease in the expression of p95HER2 compared to p185HER2, accompanied with decreased Akt expression in the JIMT-

1 cells treated with aprepitant. Considering that JIMT-1 is both a trastuzumab- and lapatinib-resistant cell line [40], aprepitant may have applications in the dual resistance to trastuzumab and lapatinib. To the best of our knowledge, this is the first study that demonstrated the downregulation of HER3 and p95HER2 with aprepitant treatment. Further in-depth studies need to be carried out in order to elucidate the exact mechanisms of how aprepitant affects HER2 signaling.

Previous studies have identified the priming effect of SP on various type of stem cells (SC), including neural SC, epithermal SC, bone marrow-derived mesenchymal SC, adipose derived SC, pluripotent tendon cells, and cardiac SC, involving cellular regeneration [41]. However, in terms of cancer treatment, the presence and increase in CSCs are major obstacles that need to be overcome in order to conquer cancers. The traditional cytotoxic chemotherapeutic agents non-selectively kill rapidly growing cells; therefore, while the quiescent CSC are resistant to therapy, they harbor the capacity to replenish a tumor after therapy. Thus, as treatment is repeated, resistant populations derived from CSC are increased, making it more difficult to treat advanced cancers that have responded poorly to chemotherapy [42]. Furthermore, aprepitant showed the ability to suppress the BCSC-like properties of HER2-positive breast cancer cells, as evidenced by a marked inhibition of ALDH1 activity, reduction of the $CD44^{high}/CD24^{low}$ subpopulation, and the impairment of mammosphere

formation. While this ability was not seen in HER2-negative breast cancer cells (MDA-MB-231), it was observed in all the HER2-positive cells evaluated, regardless of their trastuzumab resistance (JIMT-1) or hormone receptor expression (BT474) status. To the best of our knowledge, this is the first study that demonstrated the effectiveness of aprepitant in targeting BCSC in different types of HER2-positive breast cancer cells. Although the exact mechanisms used to target BCSC were not addressed in this study, a prior study using human colon cancer cells suggested that β-catenin/Wnt and Akt/mTOR were two pivotal pathways that were repressed by aprepitant, affecting the CSC-like cancer cells [43].

Overall, we demonstrated that aprepitant exerted antitumor activity in HER2-positive breast cancer cells via suppression of the HER2/Akt signaling and BCSC-like properties. These antitumor effects were also observed in the JIMT-1 cells, which derived from an actual patient with trastuzumab-resistant HER2-positive breast cancer and who also displayed lapatinib resistance, implying that aprepitant could overcome multidrug-resistance. While the precise mechanism of aprepitant's effect on cancer cells still remains to be elucidated and considerable works need to be done to identify the safe and effective dose of aprepitant required to exert anti-cancer activity and to establish an adequate treatment strategy in actual cancer patients, our findings suggested that aprepitant could be an attractive candidate for drug

repurposing as a new alternative agents for HER2-positive breast cancer patients who have experienced failure with trastuzumab treatment.

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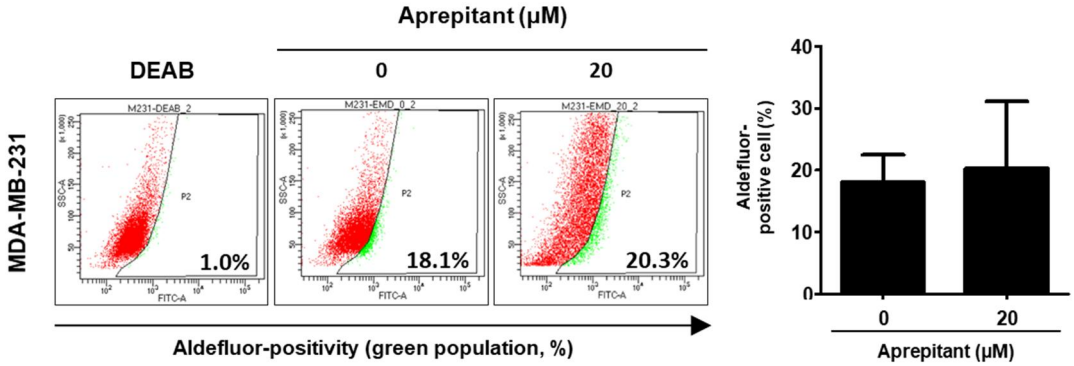
Appendix



Supplemental figure 1. The effect of aprepitant on ALDH1 activity in HER2-negative MDA-MB-231 cells.

No significant change was observed in the proportion of ALDEFLUOR-positive cells after exposure to aprepitant (20 μM, 72 h) in MDA-MB-231 triple-negative breast cancer cells. ALDEFLUOR-positive cells were analyzed by flow cytometry. As a specific inhibitor of ALDH1, 50 mM diethylamino-benzaldehyde (DEAB) was used for defining the ALDEFLUOR-positive population. Quantitative graph of ALDEFLUOR-positive cells is shown in the right panel. The results are presented as mean ± SEM of at least three independent experiments and analyzed by Student's T-test.

국문요약

유방암은 여성호르몬 수용체 양성, HER2 양성, 삼중음성유방암 3 가지로 분류하며, 그 중 HER2 양성 유방암은 전체 유방암 중 25% 정도를 차지하고 있다. HER2 양성 유방암은 일반적인 유방암에 비해 공격적인 진행 양상을 보이나, HER2 를 타깃으로 한 표적치료제가 가장 먼저 개발 되어 치료에 도입되면서 이전에 비해 환자들의 예후가 매우 향상되었다. 그러나 여전히 상당수의 환자는 적절한 치료에도 불구하고 재발을 경험하며 HER2 양성 전이성 유방암의 경우 완치가 불가능하다. 이는 근본적으로 암세포의 HER2 표적치료제에 대한 내성 발현에 기인하며 트라스투주맙으로 대표되는 HER2 표적치료제의 내성을 극복하기 위한 새로운 약제 개발을 위해 많은 연구들이 진행되고 있다.

본 연구에서는 신경전달물질인 substance P 와 그의 수용체인 뉴로키닌 1 수용체 (neurokinin 1 receptor, NK1R)의 결합이 암세포의 tumorigenesis 에 관여하며, 특히 HER2 양성 유방암에서 HER2 활성화와도 관련이 있다는 이전 연구 결과들을 바탕으로 현재 임상에서 항구토제로 쓰이고 있는 NK1R 길항제인 아프레피턴트 (aprepitant)의 HER2 양성 유방암 대상 항암효과와 트라스투주맙 내성 극복 가능성에 대해 연구하였다.

본 연구에서는 트라스투주맙 민감성 세포주인 SKBR3, BT474 와 내성 세포주인 JIMT-1 세포를 이용하여 실험을 진행하였다. 그 결과 아프레피턴트는 트라스투주맙 내성 여부와 관계 없이 HER2 양성 유방암 세포주 모두에서 세포생존율을 유의하게 감소시켰고 caspase 의존적인 세포사멸을 유도하였으며 이러한 과정에는 MEK/ERK 활성 억제가 관여한다는 것이 확인되었다. HER2 신호전달계와 관련하여 아프레피턴트는 HER2 양성 유방암 세포에서 HER3 의 downregulation 을 유도하였으며 p95HER2 의 발현도 현저히 감소시켰다. 또한

아프레피턴트는 HER2 양성 유방암 세포군 내 유방암 줄기세포 군집을 억제하는 효과를 보여주었는데 이는 세포 내 ALDH1 활성 억제 뿐 아니라 JIMT-1 세포에서 $CD44^{high}/CD24^{low}$ 표현 집단의 감소, 그리고 BT474 세포를 이용한 mammosphere 형성능 억제를 통해 증명되었다. 트라스투주맙과 아프레피턴트의 병용 처리 결과 아프레피턴트는 트라스투주맙 내성 세포에서 트라스투주맙의 효능을 증대시킬 수 있는 sensitizer 로서의 역할은 보이지 않았다. 그러나 아프레피턴트 단독 처리로도 JIMT-1 과 같은 다약제 내성 세포 (multidrug resistance)의 현저한 세포 억제 효과를 나타낸 것은 아프레피턴트가 그 자체로 트라스투주맙 내성을 극복하여 항암효과를 나타낼 수 있음을 의미한다.

암세포에 영향을 미치는 아프레피턴트의 정확한 기전은 아직 밝혀지지 않았고, 항암 효과를 발휘하는 아프레피턴트의 안전하지만 효과적인 용량을 확인하고 실제 암 환자에 대한 적절한 치료 전략을 수립하기 위해서는 상당한 연구가 필요하지만, 본 연구의 결과는 아프레피턴트가 트라스투주맙 치료에 실패한 HER2 양성 유방암 환자를 위한 새로운 대체 약제로 신약재창출 (drug repurposing)이 가능한 매력적인 후보가 될 수 있음을 시사한다.