

# Dexamethasone interferes with trastuzumab-induced cell growth inhibition through restoration of AKT activity in BT-474 breast cancer cells

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**Abstract.** The combination of trastuzumab with paclitaxel (PTX) is an important option for the treatment of HER2-positive breast cancer. Dexamethasone (Dex) premedication is routinely used in the treatment with PTX. The interactions among Dex, PTX and trastuzumab were evaluated in BT-474 cells. Dex interfered with trastuzumab-induced cell growth inhibition without clear effects on PTX-induced cytotoxicity. Trastuzumab dephosphorylated retinoblastoma protein (pRB). Dex restored this trastuzumab-induced dephosphorylation of pRB and released trastuzumab-induced G<sub>1</sub> arrest. Trastuzumab suppressed AKT activity without affecting ERK activity. A specific inhibitor for the phosphatidylinositol 3-kinase/AKT pathway, LY294002, inhibited cell growth and AKT and pRB phosphorylation. Dex restored the trastuzumab-induced suppression of AKT without affecting ERK activity. It was concluded that Dex interferes with trastuzumab-induced cell growth inhibition, at least partially, through the restoration of trastuzumab-induced AKT suppression and subsequent pRB dephosphorylation in BT-474 breast cancer cells. These observations support the development of new chemotherapeutic regimens without glucocorticoid premedication.

## Introduction

HER2 is over-expressed in 20-30% of breast cancer, and has been proposed to be related to cell growth and survival (1). A humanized IgG1 monoclonal antibody that recognizes an epitope in the extracellular domain of HER2, trastuzumab (Herceptin®) is used in the treatment of HER2-positive breast cancer (2). Clinical studies have been established showing that trastuzumab is active against HER2-overexpressing metastatic breast cancers (MBC) either in combination with

chemotherapeutic agents (3-6) or alone (7,8). Among cytotoxic drugs combined with trastuzumab, paclitaxel (PTX) is a major chemotherapeutic agent for the treatment of MBC (1). Furthermore, as post operative chemotherapy in HER2-positive breast cancer, the combination of trastuzumab with PTX is recommended after treatment with doxorubicin and cyclophosphamide (9). Accordingly, the combination of trastuzumab with PTX is an important option for the treatment of breast cancer.

In the clinical use of PTX, a hypersensitivity reaction is one of the major adverse events (10). Glucocorticoids, including dexamethasone (Dex), are used to prevent these reactions (11,12). Because Dex premedication is routinely used in the treatment with PTX, the effect of Dex on PTX-induced cytotoxicity is an important issue. In this regard, Wu *et al* reported that the treatment with Dex decreases the cytotoxicity by PTX through NF- $\kappa$ B inhibition in breast cancer cell lines (13). Similarly, we reported antagonistic effects of Dex on PTX-induced cytotoxicity through inhibition of the cell cycle progression in non-small cell lung cancer cell lines (14).

Since the combination of PTX and trastuzumab is frequently utilized in the treatment of breast cancer, trastuzumab is deservedly used concomitantly with Dex. However, there have been few findings on the interaction between trastuzumab and Dex. In the process of evaluating the effect of Dex on cell growth inhibition of the combination of PTX and trastuzumab in breast cancer cells, we found that Dex strongly interferes with the trastuzumab-induced cytostatic effect rather than with the cytotoxicity of PTX. Here, we report that Dex precludes trastuzumab-induced cell growth inhibition through activation of the AKT pathway in HER2-over-expressing BT-474 breast cancer cell lines.

## Materials and methods

**Chemicals and reagents.** Trastuzumab (Chugai Pharmaceutical Co., Tokyo, Japan) was diluted in distilled water and stored at -80°C. PTX (a gift from Bristol-Myers-Squibb, Tokyo, Japan) and 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) (Sigma-Aldrich Japan, Tokyo, Japan) were dissolved in dimethylsulfoxide and stored at -20°C. Dex (Wako Pure Chemical Industries, Osaka, Japan) was dissolved

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in dimethylsulfoxide and stored at  $-80^{\circ}\text{C}$ . 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Co., St. Louis, MO) was dissolved in phosphate-buffered saline (PBS) and stored at  $-20^{\circ}\text{C}$ .

**Cell lines and cultures.** Human breast cancer cell lines, BT-474 cells, were obtained from American Type Culture Collection (Manassas, VA). The BT-474 cell lines were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics. The cells were grown in a humidified atmosphere of 5%  $\text{CO}_2$ -95% air.

**MTT assay.** The growth inhibitory effects of these chemotherapeutic agents were estimated by an MTT assay. BT-474 cells were counted with a hemacytometer and  $6 \times 10^3$  cells were incubated in 100  $\mu\text{l}$  medium containing various concentrations of Dex, PTX and trastuzumab for 72 h using 96-well flat bottom multiplates (BD Falcon, Franklin, NJ). The plates were incubated under the same conditions for the indicated time. After the indicated treatment, MTT solution (10 mg/ml in PBS) was added (10  $\mu\text{l}$ /well). The plates were further incubated for 4 h at  $37^{\circ}\text{C}$ . Thereafter, the formazan crystals formed were dissolved by adding 100  $\mu\text{l}$  of 0.04 N HCl in 2-propanol. Absorption was measured by a microplate reader (MPR-A4i; Tosoh Corp., Tokyo, Japan) at 570 nm (reference filter 650 nm). Measurements were performed in triplicate. DMEM (100  $\mu\text{l}$ ) with 10  $\mu\text{l}$  MTT-solution and 100  $\mu\text{l}$  2-propanol was used as blank solution.

**Cell lysis, immunoprecipitation, and Western blot analysis.** The BT-474 cells were lysed in a modified radioimmune precipitation buffer (1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 2 mM EDTA, 10  $\mu\text{g}/\text{ml}$  leupeptin, 1 mM phenylmethyl-sulfonyl fluoride, 10 mM NaF, 40 mM  $\beta$ -glycerophosphate, and 2 mM  $\text{Na}_3\text{VO}_4$ ), and the insoluble material was removed by centrifugation. The protein concentration was determined by means of a Bio-Rad protein assay (Bio-Rad, CA) and lysates containing 50  $\mu\text{g}$  of total cellular protein or immunoprecipitates with the indicated antibodies were analyzed by Western blotting after SDS-polyacrylamide gel electrophoresis and visualized by enhanced chemiluminescence detection (Amersham Pharmacia Biotech) using goat anti-rabbit or anti-mouse IgGs coupled with horseradish peroxidase as a secondary antibody (Amersham Pharmacia Biotech). Antibodies against retinoblastoma protein (pRB) were purchased from Santa Cruz Biotechnology (IF8 for immunoprecipitation) and Cell Signaling Technology (4H1 for Western blot analysis). Anti-ERK1/2 and anti-active ERK1/2 (p-ERK1/2) antibodies were purchased from Promega Corp.. Anti-AKT and anti-active AKT (p-AKT) antibodies were purchased from Cell Signaling Technology. An anti- $\beta$ -actin antibody was obtained from Sigma-Aldrich Japan.

**Bromodeoxyuridine incorporation studies.** Measurement of the rate of proliferation was performed by determining nucleotide incorporation into DNA (15). The BT-474 cells were incubated for 24 h in 1 ml of medium containing 10% FBS using 60-mm dishes. After 24 h, the growth media was removed. The BT-474 cells were treated with 2  $\mu\text{M}$  of Dex, 10  $\mu\text{g}/\text{ml}$  of

trastuzumab, or their combination for 72 h. Then 10  $\mu\text{M}$  of bromodeoxyuridine (BrdU) was added into the solution 4 h before the analysis. After these treatments, the cells were washed, collected by trypsinization and re-suspended in 1-ml PBS. The cells were fixed in ice-cold 70% ethanol overnight at  $-20^{\circ}\text{C}$ . The fixed cells were washed with PBS and then mixed with 4 M HCl containing 0.5% Triton-X. After 30 min at room temperature, the cells were washed with PBS and neutralized by 0.1 M sodium tetraborate (pH 8.5). Then FITC conjugated anti-BrdU antibody was added to the cells for 2 h at room temperature in the dark. After a wash step, the cells were treated with 1 ml of propidium iodide solution (10  $\mu\text{g}/\text{ml}$  of propidium iodide, 20 mM EDTA, 0.05% Tween-20, 50  $\mu\text{g}/\text{ml}$  RNase). Cell fluorescence was analyzed by a Becton FACScan using cell Quest software (Becton Dickinson, Mountain View, CA).

## Results

**The effects of trastuzumab, PTX and Dex on the proliferation in BT-474 cells.** The BT-474 cells were treated with various concentrations of trastuzumab and PTX concurrently for 72 h and growth inhibition was assessed by MTT assay. As shown in Fig. 1A, the combination effects of trastuzumab and PTX were additive. In the clinical utilization of trastuzumab and PTX, Dex must be used to avoid hypersensitivity reactions. Hence, we investigated the effects of Dex on PTX- and trastuzumab-induced cell growth inhibition. The BT-474 cells were treated with PTX or trastuzumab for 72 h in the presence of various concentrations of Dex. Clear effects of DEX on PTX-induced growth inhibition were not observed in the combination of Dex and PTX. However, in the combination of Dex and trastuzumab, Dex interfered with the trastuzumab-induced growth inhibition in a concentration-dependent manner (Fig. 1B).

In preliminary experiments, treatments with 1  $\mu\text{g}/\text{ml}$  of trastuzumab, the combination of 0.5  $\mu\text{g}/\text{ml}$  of trastuzumab and 1 nM of PTX or 10 nM of PTX for 72 h exerted 50% growth inhibition activity in BT-474 cells. To compare the effects of Dex on trastuzumab- and PTX-induced growth inhibition, various concentrations of Dex were combined with these treatments (Fig. 1C). As shown, the effects of Dex were marginal in the treatment with PTX alone, and the interference by Dex to growth inhibition increased in the order of the treatment with trastuzumab alone, trastuzumab and PTX, and PTX alone. We concluded that trastuzumab-induced growth inhibition is affected more strongly than that by PTX in BT-474 cells.

**Effects of trastuzumab and Dex on the cell cycle progression in BT-474 cells.** For the purpose of linking cell cycle distribution and the biological response to Dex and trastuzumab, we performed DNA content analysis by propidium iodide staining using BrdU in BT-474 cells. BT-474 cells were treated with 10  $\mu\text{g}/\text{ml}$  of trastuzumab, 2  $\mu\text{M}$  of Dex or their combination for 72 h. BrdU was added during the last 4 h before the analysis. DNA ploidy analysis by flow cytometry is shown in Fig. 2A. After 10  $\mu\text{g}/\text{ml}$  of trastuzumab treatment for 72 h, cells in the  $G_1$  and S phases increased and decreased, respectively, indicating  $G_1$  arrest induced by trastuzumab.

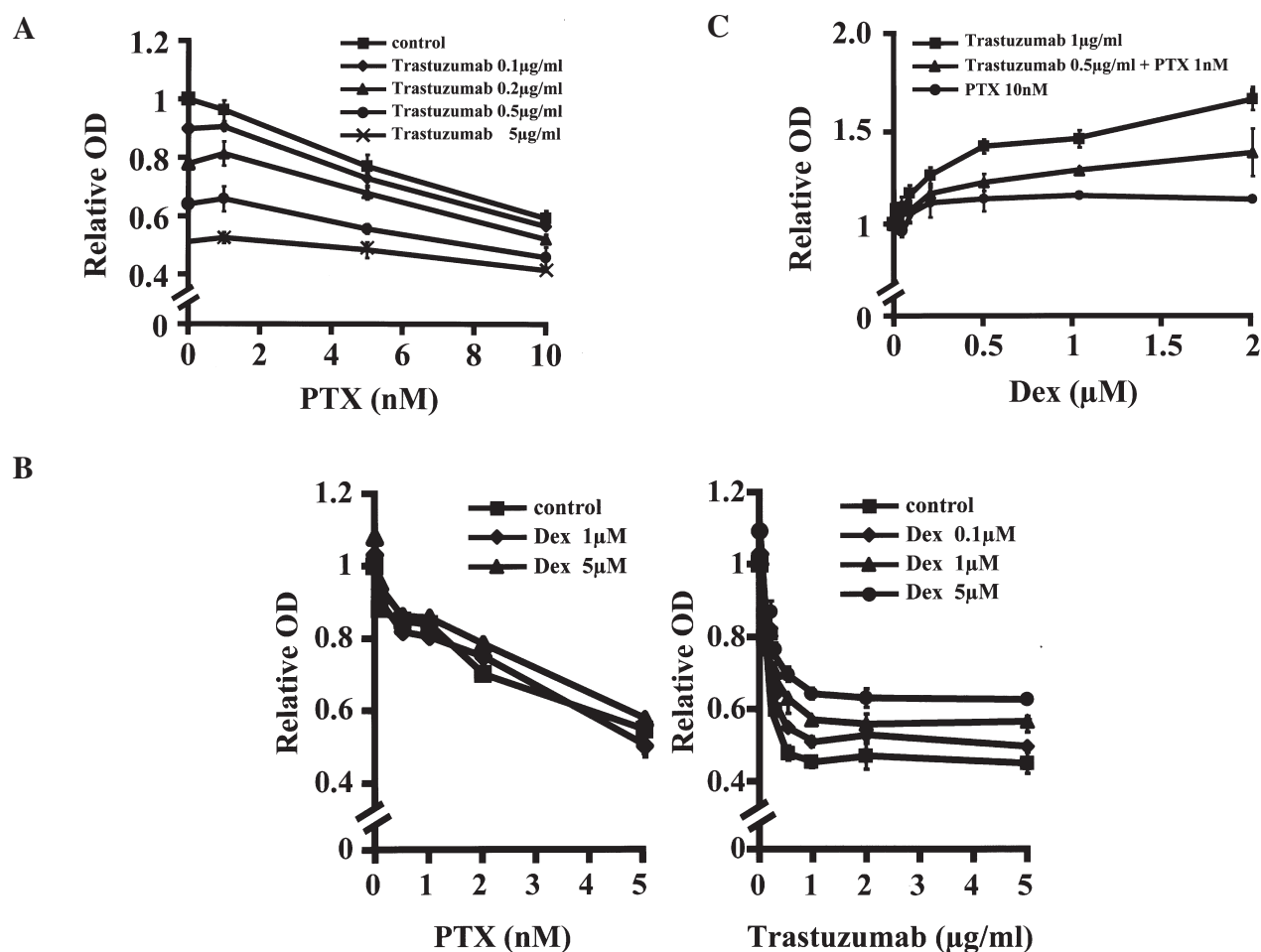


Figure 1. Interference of dexamethasone (Dex) to the trastuzumab-induced cell growth inhibition. BT-474 cells were treated with the combinations of (A) trastuzumab and paclitaxel (PTX), (B) PTX and Dex (left panel), or trastuzumab and Dex (right panel) for 72 h. The cell count was assessed by MTT assay and expressed as the relative optical density (OD) in reference to that of untreated control cells. (C) BT-474 cells were treated with various concentrations of Dex in the presence of trastuzumab (1 μg/ml) alone, trastuzumab (0.5 μg/ml) and PTX (1 nM), or PTX (10 nM) alone for 72 h. The survival cell fraction was expressed as the relative OD in reference to that of the Dex-untreated cells. Data are presented as the mean ± standard deviation of three separate experiments.

The combination of trastuzumab with Dex decreased and increased cells in the G<sub>1</sub> and S phases, respectively, compared with those of the trastuzumab alone. These results indicate that Dex interferes with trastuzumab-induced G<sub>1</sub> arrest.

To clarify the underlying biochemical mechanism by which Dex perturbs trastuzumab-induced G<sub>1</sub> arrest, we investigated the effects of trastuzumab, Dex and the combination of these agents on retinoblastoma protein (pRB) phosphorylation.

As shown in Fig. 2B, the treatment of BT-474 cells with 10 μg/ml of trastuzumab dephosphorylated pRB in a time-dependent manner. Thus, BT-474 cells were treated with 10 μg/ml of trastuzumab, 2 μM of Dex or their combination for 72 h (Fig. 2C). The pRB was dephosphorylated clearly when the cells were treated with 10 μg/ml of trastuzumab alone for 72 h. Although Dex alone did not affect pRB phosphorylation, the combination of Dex and trastuzumab restored the phosphorylation level of pRB to a considerable extent. Taken together, the interference of Dex to trastuzumab-induced growth inhibition is attributable to release of the trastuzumab-induced G<sub>1</sub> arrest through the restoration of pRB phosphorylation by Dex.

*Effects of trastuzumab and Dex on the activity of AKT and ERK in BT-474 cells.* To understand the signaling route that leads to pRB dephosphorylation by trastuzumab, the active form of ERK and AKT were evaluated by Western blotting after treatment with various concentrations of trastuzumab for the indicated time (Fig. 3A). As shown, 10 μg/ml of trastuzumab reduced AKT phosphorylation after 12-24 h, and this dephosphorylation was maintained thereafter. A concentration-dependent suppression of AKT phosphorylation was also observed. However, ERK phosphorylation did not change in the range of trastuzumab treatments tested (≤10 μg/ml of trastuzumab for 72 h) (Fig. 3A).

To confirm that the trastuzumab-induced cell growth inhibition is attributable to AKT inhibition, the effects of a specific inhibitor for the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, LY294002, on AKT and pRB phosphorylation and cell growth were evaluated. The treatment with 25 μM of LY294002 clearly inhibited AKT and pRB phosphorylation (Fig. 3B), and cell growth (Fig. 3C) in BT-474 cells.

*Effects of Dex on trastuzumab-induced AKT dephosphorylation in BT-474 cells.* BT-474 cells were treated with 10 μg/ml of

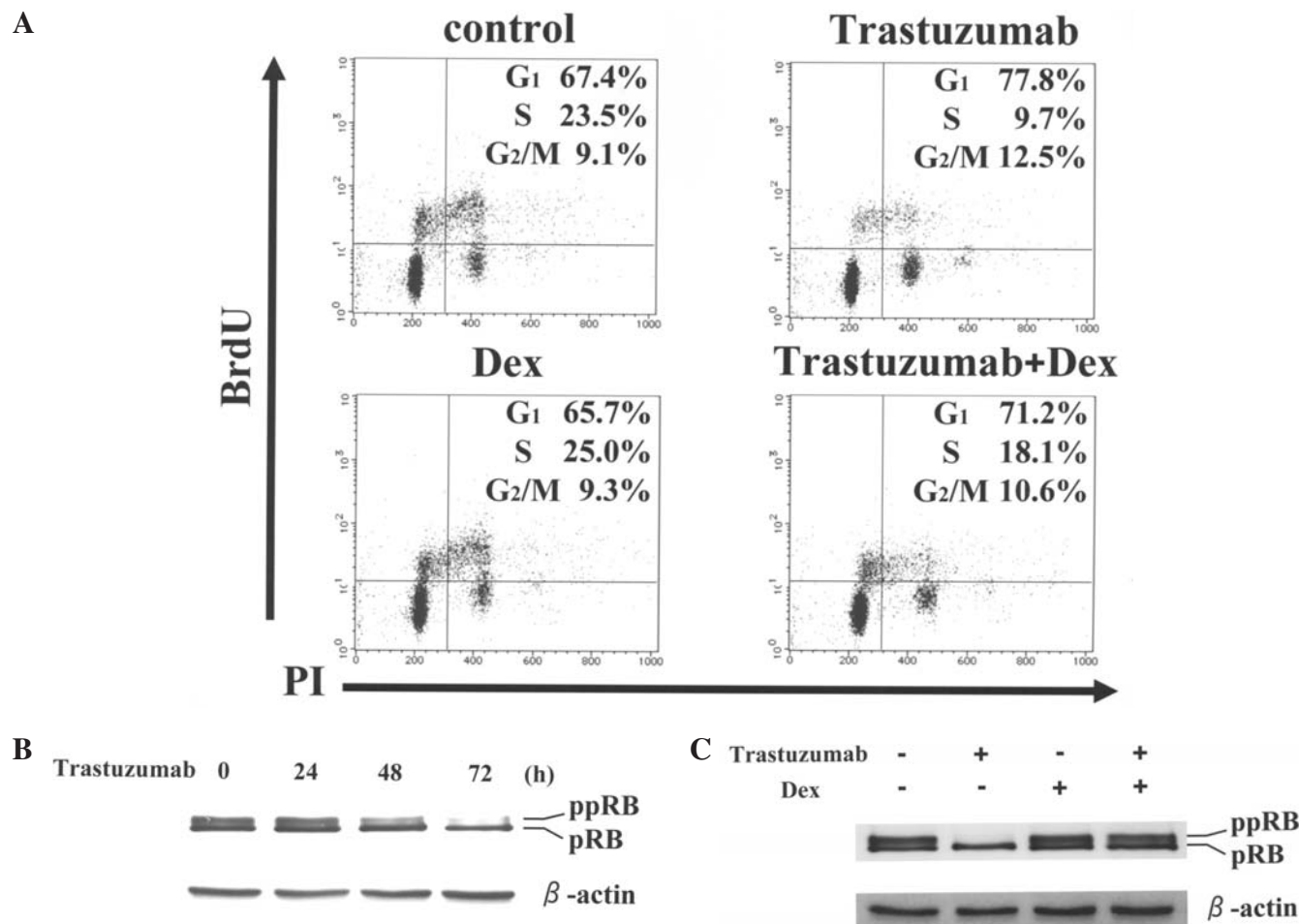


Figure 2. Effects of trastuzumab and dexamethasone (Dex) on the cell cycle progression in BT-474 cells. (A) BT-474 cells were treated with normal medium, 10  $\mu$ g/ml of trastuzumab, 2  $\mu$ M of Dex or their combination for 72 h. Ten micromolars of BrdU were added during the last 4 h before the analysis. The cells were fixed, stained and analyzed as described in Materials and methods. Representative scatter plots with the log FITC anti-bromodeoxyuridine (BrdU) staining versus propidium iodide (PI) staining are shown. The cell cycle distribution of the cells was calculated using the gates in each scatter plot and expressed as a percentage. (B) BT-474 cells were treated with 10  $\mu$ g/ml of trastuzumab for the indicated time. Retinoblastoma protein (pRB) was immunoprecipitated from 1 mg of total cellular protein by the anti-pRB antibody (IF8) and subjected to Western blot analysis with anti-pRB (4H1) as described in Materials and methods. pRB, hypophosphorylated RB; ppRB, hyperphosphorylated RB. Fifty micrograms of total cellular protein from the same cell lysate were subjected to Western blot analysis with anti- $\beta$ -actin antibody. (C) The BT-474 cells were treated with 10  $\mu$ g/ml of trastuzumab and/or 2  $\mu$ M of Dex for 72 h. Immunoprecipitate with anti-pRB antibody (IF8) or total cellular protein were subjected to Western blot analysis with the anti-pRB (4H1) or anti- $\beta$ -actin antibody, respectively. Bands corresponding to hyperphosphorylated RB (ppRB) and hypophosphorylated RB (pRB) are indicated.

trastuzumab for 72 h. Before cell harvest, 2  $\mu$ M of Dex was combined for the indicated time (Fig. 4A). As shown, the treatment with Dex restored trastuzumab-induced AKT dephosphorylation in a time-dependent manner (Fig. 4A). Subsequently, the BT-474 cells were treated with 10  $\mu$ g/ml of trastuzumab with various concentrations of Dex for 72 h. The trastuzumab-induced dephosphorylation of AKT was clearly restored by the Dex treatment in a concentration-dependent manner (Fig. 4A). Similarly, we evaluated the effects of 10  $\mu$ g/ml of trastuzumab, 2  $\mu$ M of Dex and their combination on ERK phosphorylation after 72-h treatment (Fig. 4B). A clear effect with these treatments was not observed in terms of ERK phosphorylation.

## Discussion

This *in vitro* study was carried out in order to clarify the interactions of Dex treatment on the trastuzumab-induced growth inhibition of breast cancer cells and underlying mechanisms by which Dex interferes with the action of

trastuzumab. Our observations in the present study suggest that the restoration of AKT, but not ERK, activity by Dex is an important mechanism of its antagonistic effects on trastuzumab-induced growth inhibition.

Using BT-474 cells as a model system of HER2-positive breast cancer, we examined the effects of Dex on the trastuzumab-induced growth inhibition, phosphorylation status of pRB, and the activity of ERK and AKT.

Previous studies, including our research, reported antagonistic effects of Dex on PTX-induced cytotoxicity in breast cancer and lung adenocarcinoma cells (13,14). In the present study, PTX-induced cell growth inhibition was not significantly affected by Dex in BT-474 breast cancer cells, suggesting that this antagonistic effect is cell-specific. On the other hand, we reported here the interference of Dex to trastuzumab-induced growth inhibition *in vitro*, to our knowledge, for the first time.

Initially, it was believed that trastuzumab exerts antitumor activity in HER2 positive breast cancer through antibody dependent cytotoxicity (16). Recent studies have proposed



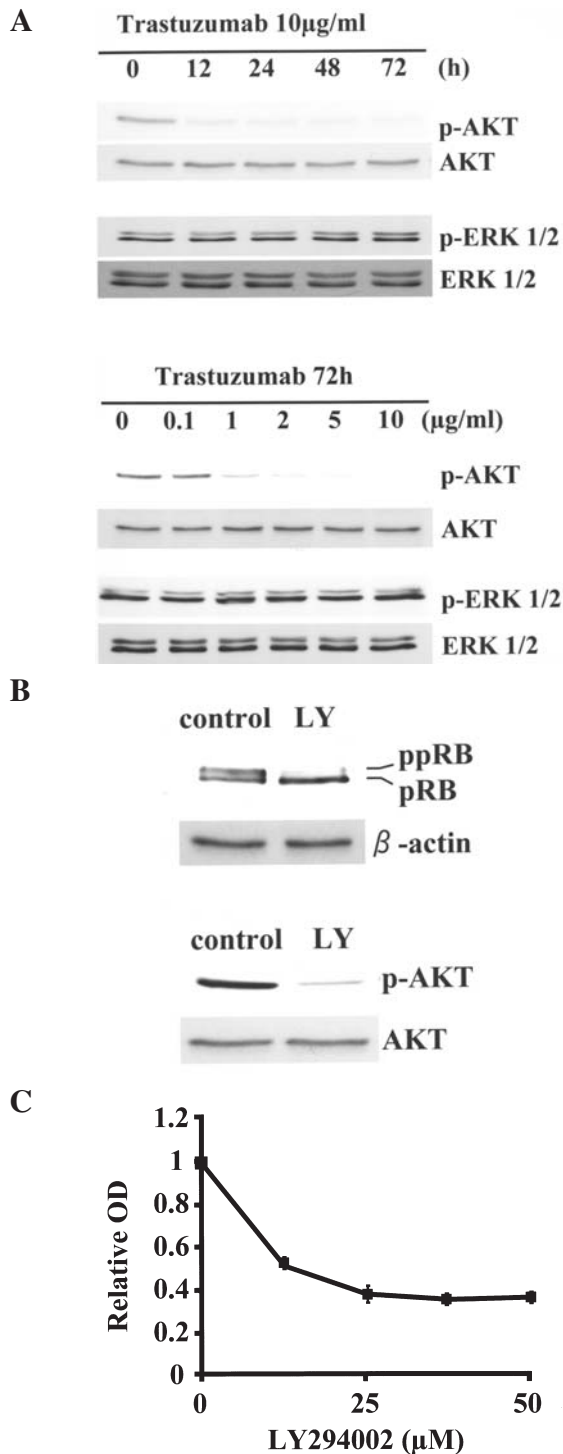


Figure 3. Effects of trastuzumab on the activity of AKT and ERK in BT-474 cells. (A) BT-474 cells were treated with 10 µg/ml of trastuzumab for the indicated time (upper panel) or the indicated concentration of trastuzumab for 72 h (lower panel). Fifty micrograms of total cellular protein from the cell lysate were subjected to Western blot analysis with anti-active AKT (p-AKT), anti-AKT, anti-active ERK (p-ERK1/2) or anti-ERK1/2 antibody as described in Materials and methods. (B) BT-474 cells were treated with 25 µM of LY294002 (LY) for 24 h. Retinoblastoma protein (pRB) was immunoprecipitated from 1 mg of total cellular protein by anti-pRB antibody (IF8) and subjected to Western blot analysis with anti-pRB antibody (4H1) (upper panel). pRB, hypophosphorylated RB; ppRB, hyperphosphorylated RB. Fifty micrograms of total cellular protein from the same cell lysate were subjected to Western blot analysis with anti-β-actin (upper panel), anti-active AKT (p-AKT) or anti-AKT (lower panel). (C) BT-474 cells were treated with various concentrations of LY294002 (LY) for 72 h. The cell count was assessed by MTT assay and expressed as the relative optical density (OD) in reference to that of untreated control cells. Data are presented as the mean ± standard deviation of three separate experiments.

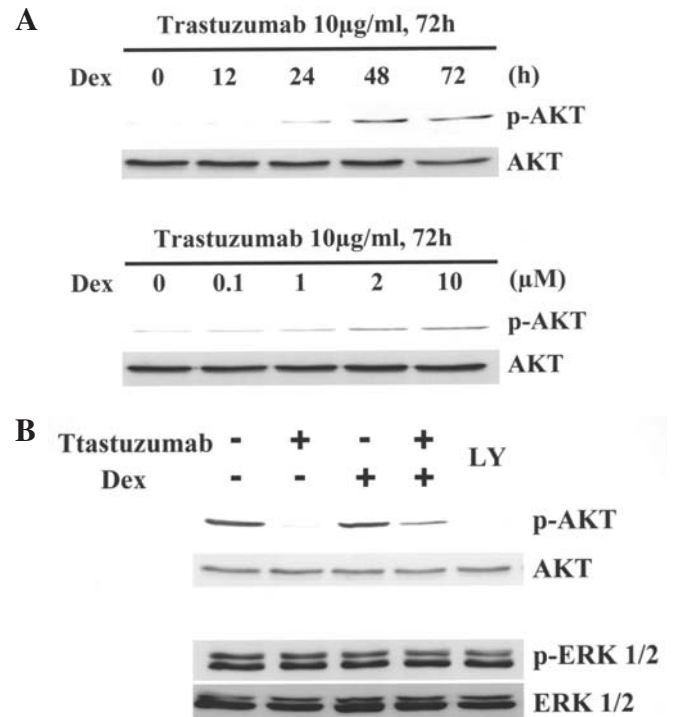


Figure 4. Effects of dexamethasone (Dex) on the activity of AKT and ERK in BT-474 cells. (A) BT-474 cells were treated with 10 µg/ml of trastuzumab for 72 h. Before cell harvest, 2 µM (upper panel) or the indicated concentration or 72 h, respectively. Fifty micrograms of total cellular protein from the cell lysate was subjected to Western blot analysis with anti-active AKT (p-AKT) or anti-AKT antibody. (B) BT-474 cells were treated with 10 µg/ml trastuzumab and/or 2 µM Dex, or 25 µM LY294002 (LY) for 72 h. Fifty micrograms of total cellular protein from cell lysate were subjected to Western blot analysis with anti-active AKT (p-AKT) and anti-AKT (upper panel) or anti-active ERK (p-ERK1/2) and anti-ERK1/2 antibody (lower panel).

that blockade of the growth promoting intracellular signal from HER2 is an additional important mechanism by which trastuzumab inhibits tumor growth (17).

In general, ERK and AKT are important mediators of cell proliferation signals from receptor tyrosine kinases to pRB phosphorylation (18). In this study, the treatment of BT-474 cells with trastuzumab inactivated AKT and dephosphorylated pRB without affecting ERK activity, in accordance with previous studies (19,20). Since an AKT inhibitor, LY294002, suppressed cell growth, AKT activity and pRB phosphorylation, trastuzumab-induced cell growth inhibition is, at least partially, attributable to a blockade of signals from AKT to pRB but not from ERK. AKT suppresses cyclin-dependent kinase (CDK) inhibitors such as p21<sup>WAF1/CIP1</sup> and p27<sup>Kip1</sup> through phosphorylation (21,22), leading to pRB phosphorylation and cell cycle progression. Thus, the inhibition of these mechanisms by trastuzumab is assumed to function in BT-474 cells.

The administration of 20-mg Dex is recommended when patients are treated with PTX (10,23). When 8 mg of Dex is injected, the concentration of Dex in peripheral blood reaches approximately 1 µM (24). Thus, we mainly assessed the effect of 2-µM Dex. This clinically relevant concentration of Dex interfered with trastuzumab-induced cell growth suppression and restored the trastuzumab-induced dephosphorylation of

AKT and pRB to some extent without affecting those in trastuzumab-free conditions. Accordingly, we concluded that the restoration of trastuzumab-induced AKT suppression and subsequent pRB phosphorylation can be regarded as a main mechanism of the antagonistic effects of Dex on trastuzumab-induced growth inhibition. To clarify the detailed mechanism, it may be necessary to assess the effects of Dex on upstream regulators of AKT such as tyrosine kinases, PI3K, phosphatase and tensin homolog (PTEN) and phosphoinositide-dependent protein kinase (PDK).

As clinical implications of interference of Dex with trastuzumab-induced cytostatic effects, Dex may attenuate the antitumor activity of the combination of trastuzumab and PTX in breast cancer. In addition, glucocorticoid may impair trastuzumab-induced antibody dependent cytotoxicity (16). In this regard, new analogue and formulations of PTX are being developed (25,26), leading to the decrease or removal of the polyoxyethylated castor oil that causes hypersensitivity reactions when using the conventional PTX preparation. Even if these drugs have the same activity against cancer cells as PTX, it may be possible for the omission of glucocorticoid pretreatment to lead to higher effectiveness of the combination treatment with trastuzumab and new generations of taxane. Our observation in this study supports this possibility, and promotes the development of chemotherapies using the new generation of taxane that obviates glucocorticoid premedication in breast cancer.

Furthermore, the observation that Dex restores trastuzumab-induced AKT suppression may have additional implications with respect to chemotherapy in which Dex is utilized as an antiemetic agent. AKT is an important mediator of anti-apoptotic intracellular signals (27). The activation of AKT confers resistance against several anticancer agents to cancer cells (28). Since our observations raise the possibility that Dex activates AKT, at least in a fraction of cancer cells, Dex might reduce the effects of some anticancer agents other than trastuzumab and PTX.

In conclusion, the present study demonstrated that the Dex treatment antagonized trastuzumab-induced growth inhibition through the activation of AKT in BT-474 breast cancer cells. These observations support the development of new chemotherapeutic regimens without glucocorticoid premedication.

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