Proteasome inhibitor bortezomib increases PTEN expression and enhances trastuzumab-induced growth inhibition in trastuzumab-resistant cells

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PTEN (phosphatase and tension homolog deleted on chromosome 10) has been shown to be inactivated in a wide range of cancers and the role of this gene product is associated with the suppression of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway in many cancers. Recently, some reports demonstrated that the degree of PTEN expression could predict trastuzumab chemosensitivity in ErbB2-overexpressing breast cancer. Here, we demonstrate the possible involvement of a proteasome inhibitor (PS341) in PTEN expression and elucidate the influence of PI3K/Akt, one of the main cascades of the ErbB2 downstream pathway, and discuss the role of the proteasome inhibitors in trastuzumab resistance. ErbB2-overexpressing SKBR3 human breast cancer cells and trastuzumab-resistant SKBR3/R cells were analyzed in this study. We show that the expression of phosphorylated Akt was highly increased in trastuzumab-resistant cells, although the expression of PI3K, phosphorylated PI3K and non-phosphorylated Akt was unchanged in comparison with wild-type SKBR3 cells. However, following treatment with PS341, the level of phosphorylated Akt was decreased in a dose-dependent manner. Conversely, the level of PTEN was increased in the same fashion. PS341 showed sufficient cytotoxicity in resistant cells in combination with trastuzumab and the efficacy of trastuzumab was inclined to be better in resistant cells under PS341 treatment. Remarkable activity of Akt was observed in trastuzumab-resistant SKBR3 breast cancer cells and this phenomenon could be associated with the decreased expression of PTEN. The proteasome inhibitor PS341 could increase the level of PTEN and inhibit the downstream pathway of ErbB2, interfering with phosphorylation of Akt. *Anti-Cancer Drugs* 17:455–462 © 2006 Lippincott Williams & Wilkins.

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Introduction

PTEN (phosphatase and tension homolog deleted on chromosome 10, also known as MMAC and TEP1) is a recently discovered tumor-suppressor gene located on chromosome 10q23.3. PTEN mutations have been implicated in a variety of human cancers including endometrial cancer (30–50%) [1,2], high-grade glioma (60–80%) [3] and prostate cancer (29%) [4], whereas homozygous deletion of the PTEN gene causes embryonic lethality [5]. PTEN also antagonizes phosphatidylinositol-3-kinase (PI3K) functions and negatively regulates Akt activities. Restoration of PTEN expression in PTEN null cells inhibits Akt activities and tumor formation [6,7].

The ErbB2 oncogene, the second member of the epidermal growth factor receptor family, encodes a transmembrane tyrosine kinase receptor. Overexpression of ErbB2, which has been seen in approximately 30% of breast cancers, is associated with poor overall survival [8]. In particular, it has been found to be associated with increased metastatic potential and resistance to chemotherapeutic agents. Several reports have implicated PI3K and Akt in ErbB2 signaling. PI3K and Akt have been shown to play an important role in proliferation and cell survival induced many cytokines [9].

Recent studies demonstrated that resistance to trastuzumab treatment is due to the level of PTEN [10–12]. Nagata *et al.* [10] demonstrated that PTEN deficiency confers trastuzumab resistance in ErbB2-overexpressing breast cancer cells both *in vitro* and *in vivo*. Furthermore, according to clinical analysis of trastuzumab efficacy and PTEN expression, they suggested that PTEN status is a unique and powerful marker for predicting clinical response to trastuzumab-based therapy.

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The ubiquitin–proteasome pathway, however, plays an important role in regulating the cell cycle, neoplastic growth and metastasis [13], and may also be critical in regulating the amount of activated signal transduction proteins and activators of transcription in various cell lines. The involvement of the proteasome in the degradation and regulation of the function of short-lived proteins, including oncoproteins, tumor suppressors and cell cycle proteins, has been extensively investigated. Considering the regulation of PTEN expression, a previous report indicated that turnover of PTEN depends mainly on proteasome-mediated degeneration in specific cell lines [14]. Here, we demonstrate that PTEN expression is attributed to trastuzumab resistance in ErbB2-overexpressing SKBR3 cells and reveal the role of the proteasome inhibitor PS341 for cell resistance through upregulating PTEN expression.

Materials and methods

Cell culture and reagents

Human breast cancer SKBR3 cells were obtained from the American Type Culture Collection (ATCC) (Manassas, Virginia, USA), maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) Fetal Bovine Albumin (FBS), penicillin (100 IU/ml) and streptomycin (100 μg/ml), and incubated in 5% CO_2. Trastuzumab-resistant SKBR3 cells (SKBR3/R) were developed by continuous exposure to trastuzumab (4 μg/ml for SKBR3/4R and 8 μg/ml for SKBR3/8R) for 4 months, during which the medium was replaced every 5 days and cells were passaged when 50% confluency was acquired. Trastuzumab resistance was confirmed by dose–response studies as described below.

Trastuzumab was purchased from Chugai Seiyaku (Tokyo, Japan) and PS341 (VELCADETM) from Millennium Pharma (Cambridge, Massachusetts, USA).

Western blot analysis

Cells ($5–10 \times 10^6$) were washed twice with PBS and then lysed with 250 μl of a hypotonic 2-[4]-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) buffer (10 mmol/l, pH 7.6) containing 50 mmol/l KCl, 0.1 mmol/l Phenylmethansulfonyfluoride (PMSF), 0.5 μg/ml aprotinin and 0.1 mmol/l sodium orthovanadate. Upon centrifugation, supernatants were adjusted to equal amounts of protein and diluted with 1 volume of 5 × SDS sample buffer and heated for 5 min at 95°C. Samples (30 g protein) were run on 4–20% SDS–PAGE gels and electroblotted onto Polyvinylidenfluorid (PVDF) membranes. Blots were blocked overnight in 5% non-fat milk powder, 0.1% Tween in PBS (blocking solution) at 4°C. Blots were then exposed to an antibody against HER2, PI3K, Akt, PTEN (Santa Cruz Biotechnology, Santa Cruz, California, USA), p-PI3K or p-Akt, (Cell Signaling, Beverley, California, USA) at a 1000-fold dilution in blocking solution (1 h, room temperature). After extensive washing with blocking solution, blots were exposed to the appropriate secondary antibody at a 10 000-fold dilution in blocking solution. After extensive washing with blocking solution, membranes were developed using the enhanced chemiluminescence detection method (ECL; Amersham Pharmacia Biotech, Piscataway, New Jersey, USA).

Determination of cytotoxicity by the MTT assay

Cells were seeded at a concentration of 3×10^3 cells/well in flat-bottom 96-well microplates. After 24 h, cells were cultivated with the appropriate cytotoxic agents for the indicated time or left untreated (trastuzumab alone: 48 and 120 h; PS341 alone: 48 h; trastuzumab + PS341: 48 h). At the end of incubation, the viability of cells was determined using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, Wisconsin, USA). The median-effect/combination index (CI) isobologram method was used for drug effect analysis.

Drug effect analysis was performed using Biosoft computer software [14,15]. Details of this methodology have been published previously [14,15]. The CI was calculated based on the most conservative assumption of mutually non-exclusive drug interactions. CI values significantly lower than 1 indicate synergy ($CI < 1$), values significantly higher than 1 indicate antagonism ($CI > 1$) and CI values not significantly higher or lower than 1 indicate additively ($CI = 1$).

Delivery of PTEN duplex short interfering RNA (siRNA) in-vitro

Duplex siRNA against PTEN (AF143314 E9: 5′-AUGCCAACAAGCUUCUUACAAUGCC-3′) and control double-stranded oligonucleotides (AF143314 E7: 5′-AUGUACCAACCGAAUCUUACAUGCC-3′) were delivered in parental SKBR3 cells (Life Technologies, Rockville, Maryland, USA). Cells were plated in 100-mm dishes at 30% confluence and transfected with oligonucleotides (10 nmol/l) using Oligofectamine (Life Technologies) 24–72 h post-plating. Cells were re-plated for individual assays described in this report 96 h post-plating. PTEN expression was determined 120 h post-plating.

Cell cycle analysis with quantification of DNA fragmentation

DNA fragmentation was measured by propidium iodide (PI) staining and Fluorescent activated cell sorting (FACS) analysis. Cells were plated on six-well plates (1×10^4 cells/well). The concentration of PS341 used for the in-vitro studies (0.1 μmol/l) was selected to ensure phosphorylated Akt inhibition and PTEN elevation with little influence of the cytotoxic effects on drug-resistant SKBR3 cells. Following incubation with 10 μg/ml

trastuzumab, 0.1 μmol/l PS341, and 10 μmol/l trastuzumab and 0.1 μmol/l PS341, cells were harvested after 48 h incubation, pelleted by centrifugation, and resuspended in PBS containing 50 μg/ml PI, 0.1% Triton X-100 Fisher Scientific, New Jersey, USA and 0.1% sodium citrate. Cells were incubated with the PI solution and flow cytometric analysis of stained cells was performed with a FACScan (Becton Dickinson, Mountain View, California, USA).

Statistical analysis

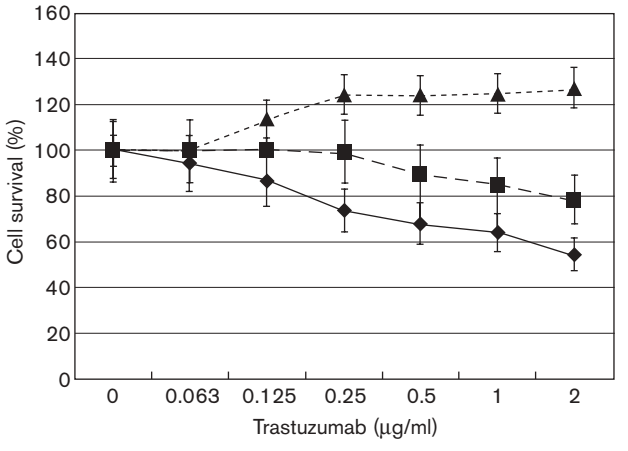
Levels of statistical significance were evaluated with data from at least three independent experiments by using two-tailed Student's *t*-test and ANOVA. $P < 0.05$ was considered statistically significant. All data were analyzed with StatView for Windows (SAS Institute, Cary, North Carolina, USA).

Results

Cytotoxicity of trastuzumab in parental and resistant cells

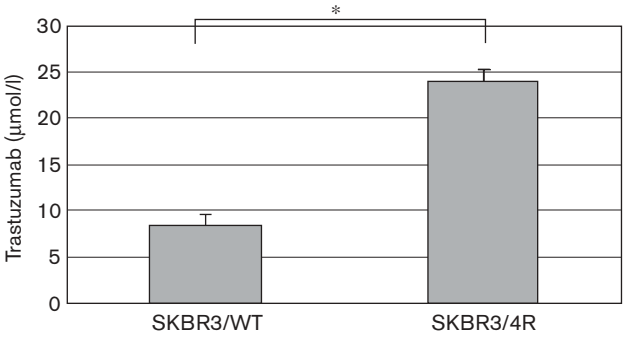
The HER2-overexpressing SKBR3 cells were treated with serial dilutions of trastuzumab over 120 h. The viabilities of cells at the various drug concentrations are shown in Fig. 1. In drug-resistant cells, mean cell viability was 79.6% (SKBR3/4R) and 128.6% (SKBR3/8R) at the concentration of 2 μg/ml of trastuzumab, and remarkably higher viability was recognized in comparison with parental cells. The IC_{50} values of trastuzumab in parental and resistant SKBR3/4R cells were 8.25 and 23.79 μmol/l, respectively, and a statistically significant difference was observed (Fig. 2).

Fig. 1



Growth inhibition assay revealed that chemosensitivity of trastuzumab was decreased in drug-resistant SKBR3/4R and SKBR3/8R cells. Diamonds = SKBR3/WT cells; squares = SKBR3/4R cells; triangles = SKBR3/8R cells. Incubation = 120 h.

Fig. 2



IC_{50}s of trastuzumab in drug-resistant SKBR3/4R cells and parental cells. The IC_{50} of trastuzumab in resistant cells was remarkably higher than that of parental cells and this difference was statistically significant. Incubation = 120 h. $^*P < 0.05$.

Cytotoxic effect of a proteasome inhibitor (PS341) alone and in combination with trastuzumab for SKBR3/WT and resistant cells

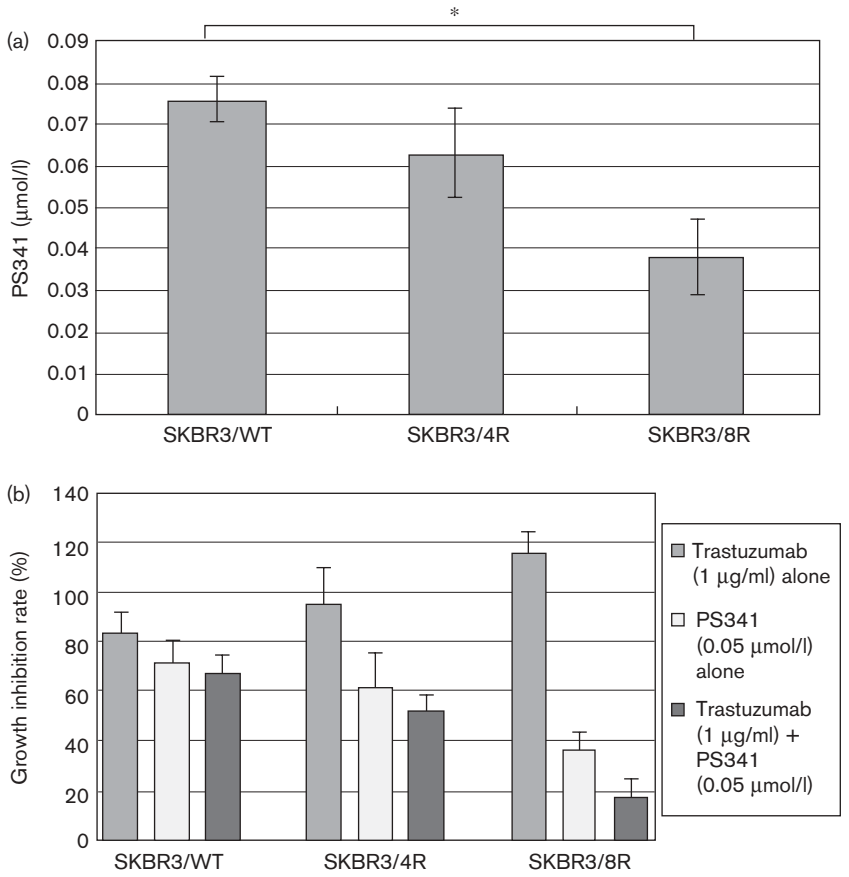
Each cell line was treated with serial dilutions of the proteasome inhibitor PS341 alone or in combination with trastuzumab. PS341 revealed sufficient growth inhibition for both parental and resistant cells, especially for resistant cells by single use. IC_{50} values of PS341 for SKBR3/WT, SKBR3/4R and SKBR3/8R were 0.076, 0.063 and 0.038 μmol/l, respectively (Fig. 3a).

In combination with PS341, trastuzumab remarkably inhibited these cells. Surprisingly, higher growth inhibition was recognized in resistant cells using a combination of both drugs (Fig. 3b).

CI of PS341 in combination with trastuzumab

To quantify the drug interactions in both resistant and parental cells, CI values of PS341 and trastuzumab were calculated. Dose–response curves were constructed for each drug and in combination at fixed molar ratios, defined as the ratio of the two agents 1:150. CI values of three separate experiments were calculated across all combinations of doses tested. A summary of the data from drug effect analysis for the drug combination in SKBR3/WT and SKBR3/4R human breast cancer cells is given in Fig. 4(a). Data points represent mean CI values, with the standard error of multiple actual experimental values and fractions of unaffected cells at the corresponding drug concentrations. Trastuzumab drug interactions in combination with PS341 were shown to be strongly dose related, with synergistic interactions ($CI < 1$) in resistant cells occurring from the lower to higher drug concentrations tested (Fig. 4a). In parental cells, however, almost additive cytotoxic effects were recognized under treatment with these chemotherapeutic agents.

Fig. 3



(a) Growth inhibition analysis of trastuzumab in SKBR3/WT cells, SKBR3/4R cells and SKBR3/8R cells in combination with specific proteasome inhibitor PS341. Growth inhibition power of PS341 was more remarkable in resistant cells after 48 h incubation with these chemotherapeutic agents. *$P < 0.05$. (b) Growth inhibition effect of PS341 is, also, remarkable for resistant cells in the concurrent treatment with trastuzumab.

PS341 increases the growth inhibition of trastuzumab in resistant SKBR3 cells and inhibits cell cycle progression

In the process of measuring the cytotoxic effects of trastuzumab (which mainly accumulates in cells in the G_0/G_1 phase), alone and in combination with PS341 (which mainly accumulates in cells in the G_2/M phase), we observed that PS341 (0.1 μmol/l) increased trastuzumab-induced accumulation of cells in the G_0/G_1 phase of the cell cycle (Fig. 4b). These effects may be associated with increased growth inhibition of trastuzumab in combination with low doses (0.1 μmol/l) of PS341 for resistant SKBR3 cells. These data suggest that PS341 could increase PTEN expression, decrease phosphorylated Akt activity and promote trastuzumab-induced accumulation of cells in the G_0/G_1 phase of the cell cycle.

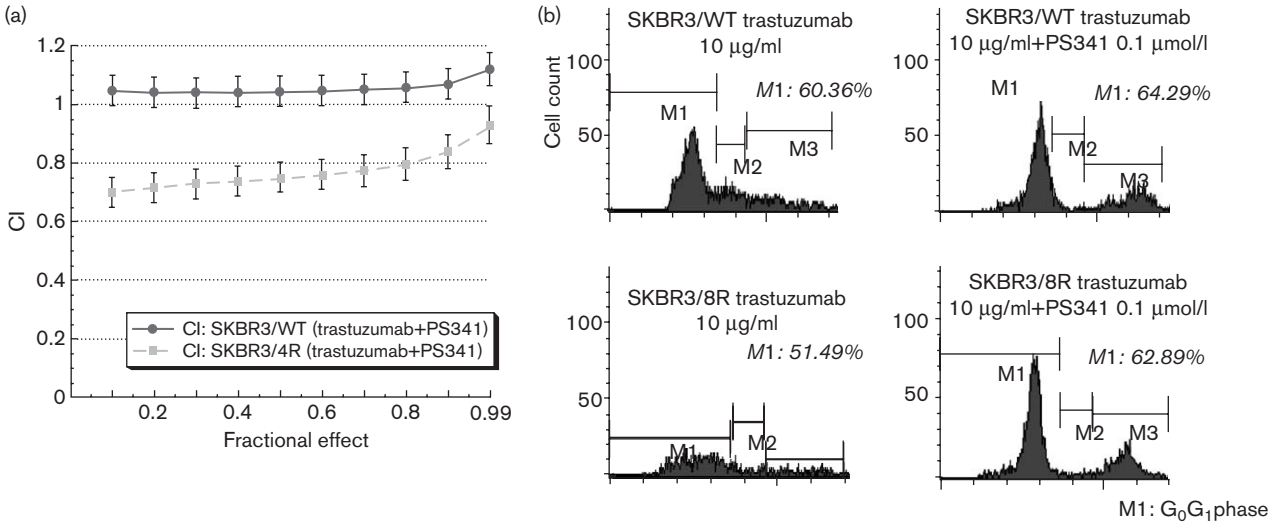
Western blotting analysis of ErbB2 expression and downstream signal proteins

Western blotting was used to detect the expression of the downstream cascade pathway of ErbB2 in both resistant and parental cells. Expression of ErbB2, PI3K, phosphorylated PI3K and non-phosphorylated Akt was the same in both resistant and parental cells. In terms of the expression of phosphorylated Akt, however, a remarkable increase was observed in resistant cells [results of densitometric scanning: Akt (SKBR3/WT = 1, SKBR3/4R = 0.95, SKBR3/8R = 1.08), p-Akt (SKBR3/WT = 1, SKBR3/4R = 2.82, SKBR3/8R = 3.83)]. Conversely, PTEN expression in resistant cells was decreased and these changes of expression were marked in more resistant cells [results of densitometric scanning: PTEN (SKBR3/WT = 1, SKBR3/4R = 0.67, SKBR3/8R = 0.18)] (Fig. 5).

PTEN deficiency and trastuzumab sensitivity in parental cells

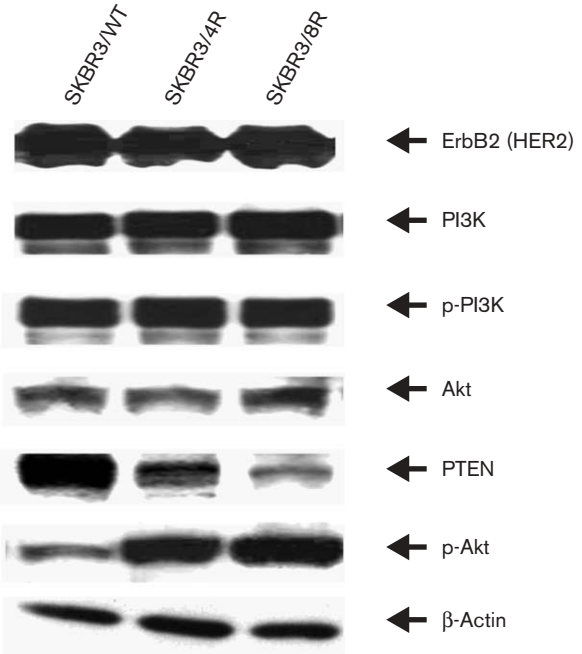
We treated SKBR3/WT cells with PTEN duplex siRNA, which effectively reduced endogenous PTEN expression compared with the cells transfected with control oligonucleotides (Fig. 6a). Compared with control cells, cells treated with PTEN siRNA showed remarkable Akt dephosphorylation by trastuzumab [results of

Fig. 4



(a) CI and fractional effects of SKBR3/4R and parental SKBR3/WT cells. Results of analysis revealed that PS341 showed additive growth inhibition power for SKBR3/WT cells in combination with trastuzumab; however, this growth inhibition power was synergistic for drug-resistant SKBR3/4R cells. (Cells were incubated with trastuzumab in combination with PS341 for 48 h and analyzed.) (b) Results of cell cycle analysis showed that, under trastuzumab treatment (10 μg/ml) the fraction of G_0/G_1 phase drug-resistant SKBR3 cells was remarkably increased in combination with PS341 (0.1 μmol/l). (Cells were incubated with these chemotherapeutic agents for 24 h and analyzed.)

Fig. 5



Results of Western blotting analysis of the ErbB2/PI3K/Akt pathway expression profile. No remarkable difference was observed for the level of ErbB2/PI3K/non-phosphorylated Akt expression in drug-resistant and parental SKBR3 cell; however, a significant decrease of PTEN expression was recognized in a drug-resistance-dependent manner. Furthermore, remarkable elevation of phosphorylated Akt was recognized in trastuzumab-resistant cells and the expression levels of phosphorylated Akt was consistent with the degree of drug resistance in these cells.

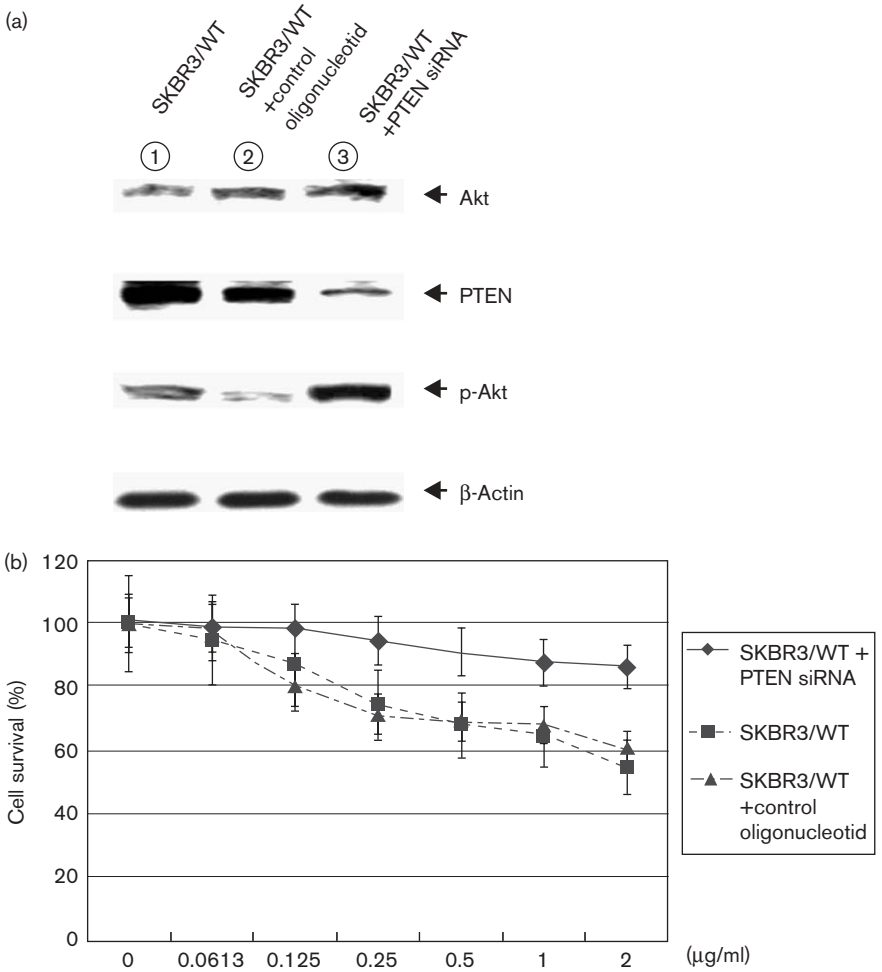
densitometric scanning: Akt (SKBR3/WT = 1, SKBR3/WT + control oligo = 1.12, SKBR3/WT + PTEN siRNA = 1.28), PTEN (SKBR3/WT = 1, SKBR3/WT + control oligo = 0.90, SKBR3/WT + PTEN siRNA = 0.19), p-Akt (SKBR3/WT = 1, SKBR3/WT + control oligo = 0.82, SKBR3/WT + PTEN siRNA = 3.74)].

To investigate whether PTEN activation contributes to trastuzumab's anti-proliferative function, we compared cell growth between control and PTEN duplex siRNA-transfected SKBR/WT cells after trastuzumab treatment. PTEN duplex siRNA-transfected cells with reduced PTEN showed significantly less growth inhibition by trastuzumab than control-transfected cells having normal PTEN expression (Fig. 6b).

Proteasome inhibitor PS341 upregulates PTEN expression and suppresses the Akt-mediated signaling pathway in drug-resistant cells

We evaluated the expression of these cascade proteins under treatment of the proteasome inhibitor PS341 in SKBR3/8R resistant cells. The proteasome inhibitor did not show any influence on the expression of ErbB2, PI3K, phosphorylated PI3K or non-phosphorylated Akt. Expression of phosphorylated Akt, however, was attenuated under the treatment of PS341 in a dose-dependent manner. Then, to elucidate the influence of PS341 on PTEN, we evaluated the expression of PTEN under the treatment of a proteasome inhibitor. PS341 increased the level of PTEN dose dependently in trastuzumab-resistant SKBR3/8R cells (Fig. 7). These results

Fig. 6



(a) Delivery of PTEN duplex siRNA showed that phosphorylation of Akt was partly due to the level of PTEN in ErbB2-overexpressing SKBR3 cells. (b) Growth inhibition curve of PTEN siRNA delivered to SKBR3/WT cells. Decreased trastuzumab sensitivity was recognized in PTEN siRNA delivered to parental SKBR3 cells. Incubation = 120 h.

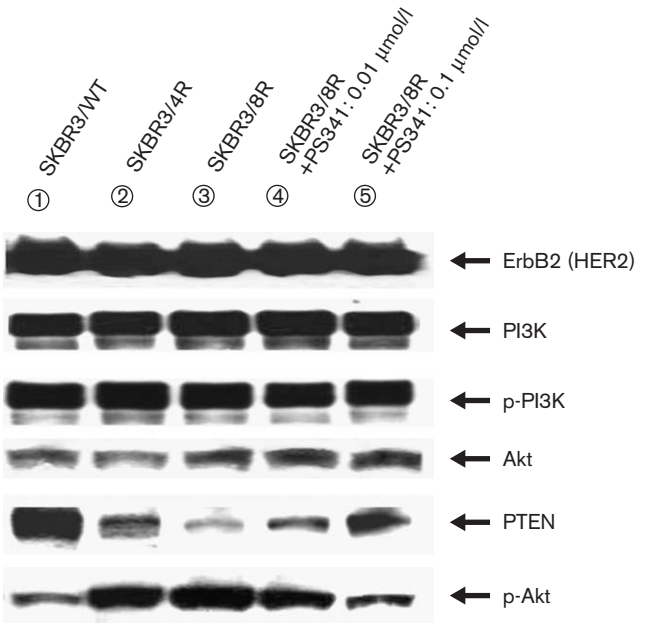
suggested that PTEN would be degraded via a proteasome-mediated mechanism and the specific proteasome inhibitor PS341 could inhibit the turnover of PTEN in trastuzumab-resistant SKBR3/8R breast cancer cells.

Discussion

Several studies using either primary tumor tissue or established tumor cell lines demonstrated a high frequency of PTEN mutation/deletion in various human cancers, including, brain, bladder, breast, prostate and endometrial cancers [1–5,15–17], and it has been found to be one of the most common targets of mutation in human cancer, with a mutation frequency approaching that of p53 [18]. In the case of human breast cancer, loss of PTEN function from PTEN mutation was reported in 5–10% of breast cancers and PTEN haploinsufficiency due to loss of heterozygosity at the PTEN locus can be found in nearly 50% of breast tumors [19,20]. In addition, epigenetic downmodulation of PTEN has also been reported [21,22].

Trastuzumab has been a model of a rationally designed, highly specific, targeted cancer therapy and has brought valuable therapeutic benefits to patients with ErbB2-overexpressing cancers [23]. In ErbB2 amplification cases, while not a cure for disseminated disease, major tumor regressions are often seen, particularly in combination with other chemotherapeutic agents. However, in spite of careful patient selection on the basis of ErbB2 fluorescence *in situ* hybridization, only limited numbers of patients respond to trastuzumab monotherapy. Therefore, these observations suggest that ErbB2 gene amplification is a necessary, but not sufficient, predictor of trastuzumab efficacy and other new predictive

Fig. 7



Expression profiles of PTEN and the ErbB2/PI3K/Akt signaling pathway. Under treatment with PS341, the phosphorylated Akt level was attenuated, alternatively and the PTEN level was increased in a dose-dependent fashion. These results suggest that the specific proteasome inhibitor PS341 could recover PTEN expression, attributed to the suppression of Akt activity, resulting in the contribution to the chemosensitivity to trastuzumab in drug-resistant SKBR3 cells.

biomarkers that determine trastuzumab responsiveness would have clinical worth in providing new strategies to improve efficacy.

Recently, some reports demonstrated that PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance *in vitro*, *in vivo* and in the clinical setting [10–12]. According to Nagata *et al.* [10], the phosphatase function of the tumor suppressor PTEN is rapidly activated by trastuzumab before it downregulates the ErbB2 receptor. This finding led to the new concept that PTEN activation is a novel mechanism that contributes to trastuzumab's anti-tumor activity independent of its well-known function of ErbB2 downregulation. This newly identified mechanism of trastuzumab anti-tumor function indicates that trastuzumab responsiveness depends not only on the downregulation of ErbB2 and inhibition of ErbB2-related downstream events, but also on the status of PTEN. In their report, furthermore, they consistently demonstrated that PTEN deficiency is a molecular mechanism that confers ErbB2-overexpressing breast cancer resistance to trastuzumab-based therapy, based on data from cultured breast cancer cell lines, mouse tumor xenografts and, most importantly, from clinical samples of breast cancer patients.

The ubiquitin–proteasome pathway plays an important role in regulating the cell cycle, neoplastic growth and metastasis [13]. A number of key regulatory proteins are temporally degraded during the cell cycle by the ubiquitin–proteasome pathway, and the ordered degradation of these proteins is required for the cell to progress though the cell cycle and to undergo mitosis. Therefore, inhibitors of the proteasome can act through multiple mechanisms to arrest tumor growth, tumor spread and angiogenesis [24–27]. The combination of these mechanisms offers a potential new approach to treating cancer and laboratory findings support this hypothesis.

The efficacy of proteasome inhibitors was vigorously investigated in hematologic malignancies and a combination of these agents was reported to enhance the cytotoxic activity of conventional chemotherapeutic agents. For instance, in the treatment of multiple myeloma, Mitsiades *et al.* [28] investigated the mechanism of the chemosensitizing activity of proteasome inhibitors with oligonucleotide gene microarray analysis. They demonstrated that a proteasome inhibitor (PS341) could downregulate the transcripts for several effectors of the protective cellular response to genotoxic stress: topoisomerase II beta, that relaxes DNA torsion on replication transcription and cell division, and is inhibited by doxorubicin [29]; the Bloom syndrome gene product, involved in the maintenance of genome integrity and stability through its cooperation with p53 [30]; and the catalytic subunit of DNA-dependent protein kinase and Ku antigen, which function in the repair of DNA double-strand breaks caused by physiologic oxidation reactions ionizing radiation and chemotherapeutic drugs [31]. Previous proteomic analysis revealed, however, that a proteasome inhibitor (PS341) decreased the expression of Bcl-2, cIAP-2, XIAP and FLIP [32].

Concerning the influence of proteasome inhibitors on PTEN, several reports indicated the role of a specific proteasome inhibitor stabilizing PTEN expression through the inhibition of proteasome-mediated PTEN degradation. Torres *et al.* [14] demonstrated that PTEN suffers rapid degradation that was diminished by the proteasome inhibitors, indicating that the turnover of PTEN in specific cells depends mainly on proteasome-mediated degradation.

In the present study, we can confirm that decreased PTEN expression would be associated with trastuzumab resistance based on the data from trastuzumab-resistant cells, and the proteasome inhibitor PS341 showed sufficient growth inhibition for both resistant and parental cells alone and in combination with trastuzumab. Moreover, in resistant cells, attenuated PTEN expression could recover to the level of parental cells under treatment with the specific proteasome inhibitor PS341

in a dose-dependent manner and increase the sensitivity of trastuzumab in resistant cells. The efficacy of trastuzumab, under the treatment of PS341, in resistant cells could therefore be partly due to the elevated expression of PTEN through the inhibition of proteasome function by PS341. The ubiquitin–proteasome-mediated degradation system, however, is widely involved in various functions of short-lived proteins and tumor-suppressor proteins. For that reason, the efficacy of PS341 in this study was naturally dependent not only on PTEN activation, but also on many other anti-tumor functions brought about by PS341.

A number of substrates have been identified for Akt kinase, including pro-apoptotic factor Bad, caspase-3 and -9, and key cell cycle modulators MDM2, p21 and p27. Furthermore, the PTEN/PI3K signaling pathway also interacts with other signaling pathways known to be essential for normal development, including the transforming growth factor-β/Smad pathway and the Wnt/β-catenin pathway. In addition to the above signaling pathways, recent studies have pointed out the role of PTEN in regulating the expression of homeobox genes, such as NKX3.1 or tumor metastasis-suppressor gene Drg-1. Taking these various mechanisms of PTEN/Akt-mediated pathways into consideration, PTEN can modulate tumor development by multiple mechanisms. Therefore, as a modulator of PTEN function, additional administration of proteasome inhibitor PS341 might offer effective treatment options for a variety of cancers together with trastuzumab treatment in ErbB2-over-expressing breast cancer.

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